Bacterial adaptation strategies and interactions in different soil habitats

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von Selma Gomes Vieira

aus Leiria / Portugal

1. Referent: Professor Dr. Jörg Overmann
2. Referent: Professor Dr. Dieter Jahn
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Huang, S., Vieira, S., Bunk, B., Riedel, T., Spröer, C. & Overmann, J. First Complete Genome Sequence of a Subdivision 6 *Acidobacterium* Strain. Genome Announc. 4: e00469-16 (2016).

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Dedicated to my late brother, Duarte Gomes Vieira

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Chapter 1 – Summary

Soil organisms play a crucial role in maintaining soil health and fertility. Of these, bacteria are the most diverse and numerous. It has been estimated that a single gram of soil contains in the order of 10^{12} prokaryotic genes and 10^{9} genomes. Bacterial communities are important in soil ecosystem functioning, particularly due to their role in soil formation, plant production and biogeochemical processes. These communities are distinct for the various niches in soil and are influenced not only by the presence of plants and other soil biota, but also by the chemical and physical properties of the soil matrix. The close and complex interplay between soil biotic and abiotic properties of soil and bacteria has major implications for agriculture, environmental and human health. Nevertheless, information regarding the functional role of these bacterial communities and their establishment over time in novel soil environments and niches is still limited.

In order to gain further insights into bacterial functions and specific interactions which mediate bacterial community development in various niches of natural temperate grasslands, culture dependent and independent approaches were employed in the present study. For bacterial communities in the rhizosphere, an overwhelming effect of soil type on rhizosphere bacterial communities was determined, significantly surpassing the influence of plants. This effect likely reflects the specific conditions of grassland where plants of different species live in very close proximity. The intermingling of root systems obviously results in a partially shared rhizosphere and the maintenance of similar rhizosphere bacterial communities. Plant species seem to influence bacterial communities to a limited degree through differences in composition of root exudates, selecting for distinct bacterial groups. For bacteria colonizing soil minerals *de novo*, mineral properties were identified as the major driver of bacterial community composition compared to the effect of different carbon sources available. Minerals provide a selective environment which counteracts the homogenising effects of dispersal, enabling the establishment of adapted bacterial communities which develop consistent temporal patterns of successional change. Nevertheless, these patterns are not consistent for bacteria of the same species or genera, since closely related bacteria displayed distinct behaviour. This lack of knowledge on bacterial functions and ecological relevance at high taxonomic resolution is only resolved with bacterial cultivation and isolation. Therefore, different cultivation approaches were applied in order to retrieve novel, relevant representatives of oligotrophic soil bacteria. Indeed, a large number of novel bacterial isolates could be retrieved, many belonging to underexplored lineages which likely harbour novel traits and functions. The novel isolate HEG_-6_39^{T} represents a novel genus of subdivision 6 *Acidobacteria*

which is highly abundant in soils. This isolate, named *Luteitalea pratensis*, was characterized and validly described.

This study provides a deepened understanding of the drivers of bacterial community composition and function in distinct niches of soil ecosystems and thereby improves our understanding of the role of bacteria in the process of soil formation and in the association with higher organisms. Ultimately, these findings have implications for a better understanding of the determinants of soil fertility.

Chapter 2 – Introduction

2.1 – Bacteria in the soil environment

Soil forms the outermost layer of the terrestrial system and represents the most complex environment on Earth, providing a wide range of habitats which are shaped by an interplay of geology, climate and vegetation. This environment is comprised of mineral particles, organic materials, pore spaces, plant roots and organisms which are distributed in a very heterogeneous way horizontally and vertically through the soil profile (Figure 1). Soil harbours a high biodiversity, and it has been estimated that the total fresh weight mass of organisms in a temperate grassland soil is higher than 45 tonnes per hectare, which equals or outweighs that of above-ground biomass (Ritz *et al.* 2003).

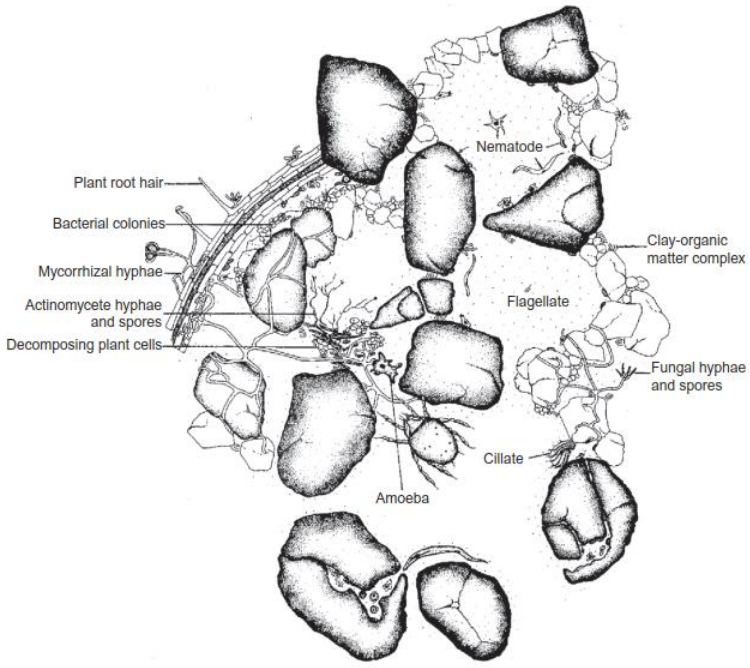

Figure 1 – Representation of a plant root and associated biota in an area of approximately 1cm^2 of soil (Frey 2015).

2.1.1 – Roles and significance of bacteria in soils

Bacteria are the most diverse and generally the more abundant constituents of soil biota and it has been estimated that a single gram of soil contains in the order of 10^{12} prokaryotic genes and 10^9 genomes (Prosser 2015). Although bacteria are frequently reported as more abundant, in some soils fungi can comprise more biomass (Sakamoto & Oba 1994; Ananyeva *et al.* 2006). One of the most important functions of soil bacteria is their contribution to nutrient cycling. The long evolutionary history of bacteria has allowed them to develop of acquire a broad range of biochemical capabilities

to recycle nearly all natural complex molecules to their inorganic constituent, which are essential to the nutrition of higher organisms. In many ecosystems, plants nutrients are those recycled through microbial metabolism, rather than through fresh nutrient inputs (Morris & Blackwood 2015). Microorganisms, and particularly bacteria, therefore are essential for plant productivity and ecosystem sustainability. This role is particularly important for carbon (C), nitrogen (N) phosphorous (P), and sulfur (S) cycling, as these comprise the major important chemical elements supporting all life on Earth. Soils are the largest terrestrial carbon reservoir, storing more carbon than the atmosphere and vegetation combined (Brevik *et al.* 2014). The flux of C from terrestrial systems is determined by relative rates of autotrophic versus heterotrophic respiration. The role of saprotrophic bacteria and fungi in the carbon cycle is the mineralization of carbon from organic material (formed by primary producers such as plants) which is then released back to the atmosphere mainly as CO_2. Phototrophic bacteria on the other hand can fix the atmospheric CO_2 back to organic carbon through photosynthesis.

The fluxes within the nitrogen cycle are primarily driven by N fixation (the conversion of atmospheric N to ammonia or other molecules accessible by organisms), by mineralization of organic nitrogen from plant and animal material (through the processes of ammonification and nitrification) and by losses of gaseous nitrogen compounds through denitrification and ammonia volatilization (Morris & Blackwood 2015). Nearly all of these processes are controlled by microorganisms, where prokaryotes are of special importance since they are the only ones capable of nitrogen fixation and are also the most important soil nitrifiers and dinitrifiers. Plants require inorganic nitrogen sources for growth but cannot use atmospheric N_2, relying instead on bacteria for a steady supply of ammonium and nitrate. Since nitrogen is the limiting nutrient in most of the soils, symbiotic relationships are often a selective advantage, like the well-known *Rhizobium*-legume association.

Phosphorous is abundant in soils, but despite the existence of this large reservoir the amount of plant-available P is usually low. Plants can only take up phosphorous in two inorganic soluble forms (hydrogen or dihydrogen phosphate) whereas most of soil P is found in insoluble forms due to its adsorption to soil surfaces and its tendency to precipitate with calcium salts in alkaline soils (Ahemad & Kibret 2014; Kertesz & Frossard 2015). Bacteria are able to transform phosphorous in two ways. The first is through mineralization of organic P (which occurs mainly as phosphate esters) to inorganic P with phosphatase enzymes. The second is through the solubilization of P in a process mediated by the production of organic acids (Aislabie *et al.* 2013).

Additionally, bacteria facilitate the uptake of iron and trace elements through the secretion of siderophores.

2.1.2 – Soil heterogeneity and bacterial hotspots

Soil consists of many habitats which can be considerably different even when only at millimetres or micrometers apart (Fierer 2017). The shape and arrangement of soil minerals and organic particles creates a network of pores of various shapes and sizes, which results in a highly uneven distribution of water, oxygen and solutes (Figure 1; Frey, 2015; Nunan *et al.*, 2007). The formation of aggregates is a determinant of soil structure, with physical forces such as drying and rewetting, and mechanical activity of soil biota shaping the organo-mineral complexes into larger aggregates (Aislabie *et al.* 2013). Despite the high amount of bacteria in soils, the complexity of the aggregate matrix prompts bacterial localization to be concentrated to small microhabitats (Vos *et al.* 2013; Kuzyakov & Blagodatskaya 2015). This spatial separation is thought to play a central role in shaping microbial community structure and functions such as maintenance of high levels of bacterial diversity, horizontal gene transfer and community stability (Raynaud & Nunan 2014). Bacteria thrive in pores where water is accessible, and microbial activity is maximized when about 60% of the pore is filled with water (Frey 2015). When moisture drops, the connectivity between pores is lost and dissolved nutrients are not easily available. On the other hand, in pores saturated with water, oxygen is the limiting factor due to lower diffusion rates, leading to the development of anoxic areas (Frey 2015). Within the aggregates, most bacteria adhere to the surface of soil particles where they form microcolonies and establish biofilms, though they only colonize a small fraction of the soil surface area, estimated at 10^{-6} % (Aislabie *et al.* 2013; Vos *et al.* 2013).

Areas with faster process rates (e.g. increased organic matter (OM) turnover, nutrient mobilization) and more intensive interactions, when compared to average soil conditions, are defined as microbial hotspots. Hotspots comprise small volumes of the soil but contribute most of the microbial mediated biogeochemical transformations, largely due to the higher availability of easily degradable organic carbon sources. Four types of microbial hotspots have been proposed, namely the rhizosphere, detritusphere, biopores and aggregate surfaces (Beare *et al.* 1995; Kuzyakov & Blagodatskaya 2015).

The *rhizosphere* is the narrow area surrounding, and influenced by, plant roots. The recruitment of bacteria to this area is initiated by the release of large amounts of organic compounds by the plant roots in a process termed rhizodeposition (Bulgarelli *et al.* 2013; Philippot *et al.* 2013). As a result, bacteria in the rhizosphere are present in much higher numbers than in the surrounding bulk soil, but these communities are less diverse (Smalla *et al.* 2001; Dennis *et al.* 2010; Peiffer *et al.* 2013). The *detritusphere* is the area where decomposition of plant and animal material takes place and encompasses the litter itself and adjacent soil influenced by it (Poll *et al.* 2006). As is the case for

rhizosphere, the detritusphere is characterized by high concentrations of easily available compounds, in early stages of decomposition as water-soluble compounds, and later as recalcitrant highly polymeric organics which then require specific enzymes and thus are decomposed at slower rates (Marschner *et al.* 2012; Loeppmann *et al.* 2016). *Biopores* are continuous large pores that are formed by soil fauna activity or were left after roots have decayed (Stirzaker *et al.* 1996; Wuest 2001). From all soil fauna, earthworms are the producers of the majority or biopores, by applying radial pressures to enlarge the burrow diameter or by moistening the soil with saliva and then ingesting it (Oades 1993). The walls of these pores have a high concentration of organic matter when compared to bulk soil and an associated higher bacterial concentration (Kirchmann & Gerzabek 1999; Brown *et al.* 2000). Bacteria associated with *aggregate surfaces* are often embedded in a mucilage which attaches to clay particles. The biofilm may provide protection against desiccation, predation, bacteriophage attack and digestion by earthworms (Frey 2015). The organic matter available for consumption in these areas is leached from the detritusphere, rhizosphere and, in deeper layers, from the A horizon above. Since all other hotspots occur mostly close to the soil surface, the aggregate surfaces are the most important hotspot in the deep soil horizons (Kuzyakov & Blagodatskaya 2015).

2.1.3 – Determinants of bacterial community structure in soil

Understanding the soil bacterial community structure is essential to elucidate the bacterial mediated processes in soil. This is a complex question as the taxonomical and functional distribution of soil bacterial communities is influenced by a multitude of factors. Persistent abiotic stressors, a high degree of competition, frequent disturbances and uneven distribution of resources (see above) severely limit soil microbial survival and growth (Fierer 2017).

Soil properties such as soil type (Girvan *et al.* 2003; Berg & Smalla 2009; Jangid *et al.* 2010), pH (Lauber *et al.* 2008, 2009; Rousk *et al.* 2010), particle size (Sessitsch *et al.* 2001) and soil moisture (Fierer *et al.* 2003; Brockett *et al.* 2012) have been identified as drivers of bacterial communities. So far, pH is regarded as one of the most important factors. In a recent study, looking at the effects of different fertilization schemes over 7 years on soils of different pH but of the same soil type, diversity and structure of bacterial communities were mostly shaped by pH rather than the nutrient additions (Zhang *et al.* 2017). Similarly, analysis of the bacterial community structure on 98 samples collected from different soils from a wide array of ecosystem types in North and South America identified pH by far as the best predictor of bacterial richness and diversity, and the strongest predictor of overall community composition (Fierer & Jackson 2006).

Different soil microbial groups have been demonstrated to have distinct substrate preferences, so it is not surprising that nutrient availability, quality and stoichiometry shape soil bacterial communities. It has been shown that taxa belonging to *Proteobacteria* and *Actinobacteria* respond positively to additions of labile organic substrates, while taxa that respond to more chemically recalcitrant substrates are fewer and phylogenetically dispersed (Goldfarb *et al.* 2011). Recently, in a study covering 179 locations and six ecosystem types across Scotland, it was demonstrated that variation in soil resource stoichiometry (total C:N:P ratios) was the primary driver of bacterial diversity. Secondary drivers included climate, soil spatial heterogeneity, soil pH, root influence and microbial biomass (Delgado-Baquerizo *et al.* 2017). Climate variables are also determinants of soil bacterial diversity. Temperature is an especially good predictor, with bacterial communities in cold regions (such as the Arctic and Antarctic) differing substantially from temperate and tropical regions. In a current analysis of bacterial diversity along a transect covering tropical to cold temperate forest soils, the mean annual temperature and mean annual precipitation were the best predictors of bacterial community structure. Soil pH and potential soil organic matter decomposition rate (SOM_{min}) emerged as the most important edaphic drivers (Tian *et al.* 2017). Furthermore, aboveground flora has a big impact in belowground biota, and significant differences exist between forests, agricultural fields, (semi-)natural grasslands and arid soils, even when these are located in close proximity (Chim Chan *et al.* 2008; Schlatter *et al.* 2015; Kaiser *et al.* 2016; Trivedi *et al.* 2016; Zeng *et al.* 2016).

It is challenging to discern individual contributions of individual parameters, at they are partially interdependent, and it is virtually impossible to measure all these factors in one experiment. Nine major components have been identified to influence changes in soil bacterial community structure (Figure 2).

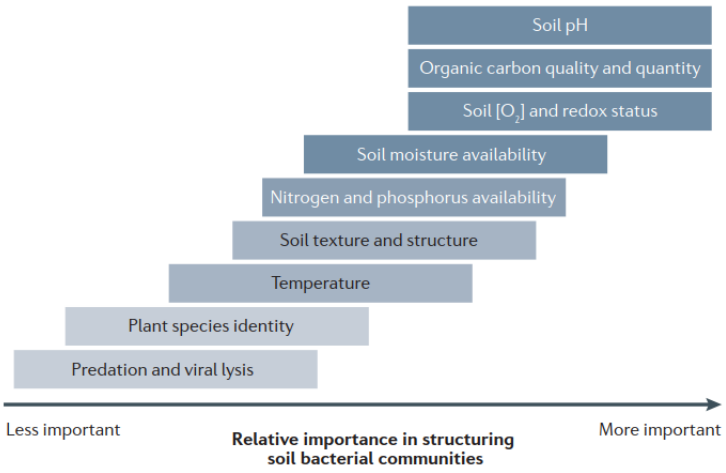

Figure 2 – Hierarchical representation of the biotic and abiotic factors that can influence soil bacterial communities (Fierer 2017).

2.2 – Bacterial association with plant roots

The association between plants and microbes is crucial for the adaptation of plants to the environment. Plants have evolved alongside their microbes and this interaction played a key role in the development of fertile land from protosoils (Reinhold-Hurek *et al.* 2015). Due to this interdependency plants and their accompanying microbiome are considered as a "holobiont", which acts as a consortium and a unit of selection in evolution (Minz *et al.* 2013). Specifically, plant growth promoting rhizobacteria (PGPR), which are rhizosphere derived organisms, positively influence plant growth in different ways. Diverse genera have already been subject of studies, mainly *Azospirillum*, *Gluconacetobacter*, *Pseudomonas* and *Rhizobium*, but many more are still to be identified (Bulgarelli *et al.* 2013).

Bacterial characteristics known to be required for efficient colonization of the rhizosphere comprise motility, lipopolysaccharide (LPS) production, capacity to form biofilms, chemotaxis and fast growth rates (Lopes *et al.* 2016). Rhizosphere bacteria also improve plant growth by providing plants with a first line of defence against soil borne pathogens. Mechanisms include antibiosis, competition for (micro-) nutrients, parasitism, interference with quorum sensing mechanisms of pathogens and induced plant systemic resistance (Mendes *et al.* 2013). This protective effect is evident in so-called "disease-suppressive soils" which are soils in which plants do not suffer from diseases (or severity is decreased) although being susceptible to them, and even though the presence of the pathogen is detected (Berendsen *et al.* 2012; Schlatter *et al.* 2017). Various secondary metabolites are produced by rhizobacteria, which inhibit the growth and activity of competing microorganisms. The excreted bacterial compounds identified to date include broad-spectrum non-ribosomally synthesized antibiotics, lytic enzymes (lysozymes), metabolic by-products such as organic acids, proteinaceous exotoxins, and bacteriocins (Subramanian & Smith 2015). One well studied example is the production of the antifungal compound 2,4-diacetylphloroglucinol (DAPG) by *Pseudomonas* spp., which inhibits the growth of the fungus *Gaeumannomyces graminis* var. *tritici* and therefore prevents the development of take-all disease in wheat (Raaijmakers & Weller 1998).

Rhizosphere bacteria are also capable of modulating the immune system of their host. Upon pathogen attack, systemic resistance response is triggered by bacteria, which primes the plant for activation of several cellular defence mechanisms, like oxidative burst, production of secondary metabolites or reinforcement of the cell wall (Philippot *et al.* 2013). In many cases this response is regulated by the phytohormones jasmonic acid, salicylic acid and gaseous ethylene (Lebeis *et al.* 2015). In exchange for all the benefits received from bacteria, plants invest a significant amount of

energy and resources in the exudation of a multitude of compounds that recruit and sustain rhizosphere bacteria.

2.2.1 – Rhizosphere effect

Physicochemical changes on the area surrounding roots make rhizosphere a selective environment on which specific bacterial communities develop (Figure 3) (Lopes *et al.* 2016). Plants influence the rhizosphere environment by altering soil pH (which can increase or decrease by up to two units) through the release and uptake of ions, by affecting soil oxygen pressure through water uptake and root respiration and by modifying soil nutrient availability through plant uptake and secretion of chelators (Philippot *et al.* 2013). Additionally, plants roots release rhizodeposits, which include a wide variety of substances that include sloughed-off root cells and tissues, mucilages, volatiles, soluble lysates and exudates, that are used as carbon sources by rhizosphere bacteria (Hirsch *et al.* 2013). This chemical, physical and biological differentiation of rhizosphere soil when compared to the remaining soil is called the "rhizosphere effect" (Massaccesi *et al.* 2015). The rhizosphere is defined by its function rather than its geometry and therefore can vary with space and time. The radial dimensions of rhizosphere can reach several millimetres in diameter for soluble nutrients or volatiles, but is less than 1 mm in the case of soluble nutrients (Minz *et al.* 2013).

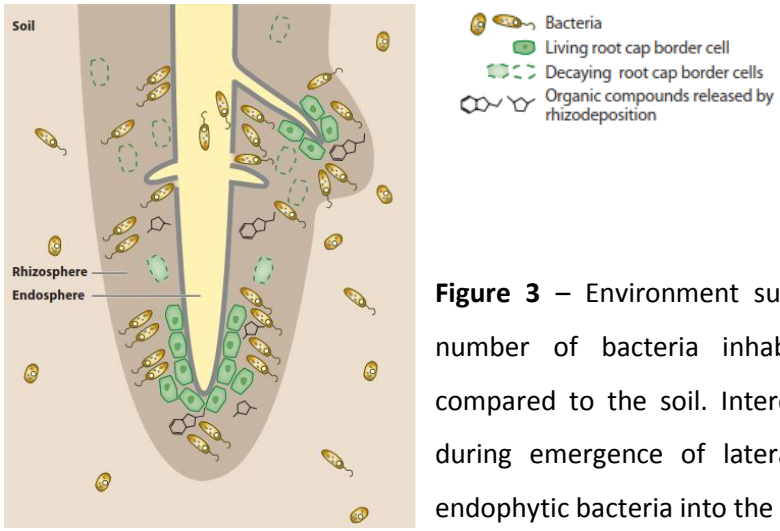

Figure 3 – Environment surrounding a plant root. A higher number of bacteria inhabit the rhizosphere area when compared to the soil. Intercellular voids on the root surface during emergence of lateral roots provides a gateway for endophytic bacteria into the root (Bulgarelli *et al.* 2013)

Rhizosphere bacterial communities are affected and shaped by many factors. Soil provides a highly diverse set of bacteria that is based on edaphic and environmental properties and affected by the history of vegetation (Reinhold-Hurek *et al.* 2015). Bulk soil can therefore be viewed as a reservoir from which rhizosphere bacteria derive. From this pool, plants are thought to select which bacteria can grow and thrive in the rhizosphere (Philippot *et al.* 2013).

Rhizosphere bacterial communities are linked to plant species, cultivar, age, health and developmental stage (Berg *et al.* 2014). Plant species-specific bacterial communities are established due to differences in root architecture and different amounts and types of rhizodeposits. A recent and broad study with 19 herbaceous plant species determined that only 1% of bacterial operational taxonomic units (OTUs) responded positively to a specific plant species, the majority of which belonged to the rare biosphere (Dawson *et al.* 2017). When investigating the rhizosphere bacterial communities of crops (wheat, oat and pea) using metatranscriptomics, differences were also identified. Rhizosphere of cereals were enriched with cellulose degraders, while the legume rhizosphere was enriched for H_2 oxidizers. H_2 is a by-product of N_2 reduction by nitrogenase and diffuses to the soil from the root nodules of legumes (Maimaiti *et al.* 2007; Turner *et al.* 2013). Another study focused on the differences between legumes and grasses, has reported that for the three legume species investigated there was an enrichment for *Actinobacteria*, *Gamma-* and *Betaproteobacteria,* and *Nitrospira*, which contrasted to the three grass species analysed which selected for *Sphingobacteria* (Zhou *et al.* 2017). Contrary to these studies, a survey of two native arcto-alpine plants (*Oxyria digyna* and *Saxifraga oppositifolia*), found no conspicuous differences between plant species with regards to their rhizosphere and endosphere bacterial communities (Kumar *et al.* 2017).

There is evidence that even within the same plant species, the host genotype can select for different assemblages of bacterial communities. This has been shown for three potato cultivars grown in two distant sites, with 4% of bacterial OTUs being cultivar specific (in both sites), belonging mostly to *Pseudomonadales*, *Actinomycetales* and *Enterobacteriales* (Weinert *et al.* 2011). Also for two wheat cultivars grown on two different fields, 24 OTUs representing 2.4% of the total rhizosphere sequences, were found to be significantly enriched or depleted on a specific wheat cultivar (Mahoney *et al.* 2017). Nevertheless, these effects are not always evident, as shown by a study using terminal restriction fragment length polymorphism (T-RFLP) on 24 different wheat cultivars. Here no significant effect of genotypes on the structure and diversity of the soil bacterial community at vegetative stage were observed (Corneo *et al.* 2016).

Another strong determinant of rhizosphere community structure is plant developmental stage, as has been shown for *Arabidopsis thaliana* at seedling, vegetative, bolting and flowering stages. Particularly, bacterial communities in the seedling stage were significantly distinct from other developmental stages. Also, metatranscriptomic analysis of the mRNA revealed that transcripts more abundant at early stages correspond with bacteria involved in providing N to plants, while at bolting and flowering stages the functional genes expressed align with PGPRs such as *Bacillus licheniformis*, *Burkholderia ambifaria* and symbiotic N-fixing bacteria (Chaparro *et al.* 2014). Similar

studies on the rhizosphere of *Avena fatua* show a succession of rhizosphere bacterial communities with plant development. A reduction of bacterial diversity is observed with time, accompanied by a reduction in the relative abundance of *Acidobacteria*, *Actinobacteria* and *Firmicutes*. Also for the rhizosphere of High Andes potato, significant effects of vegetation stage in the community composition of rhizosphere bacteria were detected, with higher relative abundances of *Actinobacteria* at flowering stage, enrichment of *Sphingomonadaceae* and *Bacteroidetes* at senescence phase and higher numbers of *Bacilli* in emergence stage (Shi *et al.* 2015).

2.2.2 – Root exudation

Around 11% of all net photosynthetically fixed carbon and 10-16% of total plant nitrogen is released into the rhizosphere by the process of rhizodeposition (Bulgarelli *et al.* 2013). Root exudates comprise the largest part of non-volatile rhizodeposits and can be grouped in two classes: low-molecular weight compounds such as aminoacids, organic acids, sugars, phenolic compounds and other secondary metabolites, and high molecular weight compounds such as polysaccharides and proteins (Dennis *et al.* 2010; Huang *et al.* 2014). The quality and quantity of root exudates is highly variable and depend on plant species/cultivars, the physiological stage of the plant, presence or absence of neighbouring plants, plant nutritional status, mechanical impedance, sorption characteristics of the soil and the rhizosphere microbial activity (Quiza *et al.* 2015). Though continuously replenished by the root, their concentration inside plant roots is much greater than in the rhizosphere due to the continuous uptake by soil microorganisms (Haichar *et al.* 2014).

The primary mechanism by which plant roots secrete low molecular weight organic compounds is a passive process mediated through three pathways: ion channels, vesicle transport and, most importantly, diffusion (Badri & Vivanco 2009). Small polar and uncharged molecules are transported by direct passive diffusion, which depends on membrane permeability and cytosolic pH (Huang *et al.* 2014). Non-polar molecules can pass through the membrane without channels or transfer proteins due to their hydrophobic nature. Polar molecules and ions can diffuse through the membrane due to facilitated diffusion, using channel proteins or permeases. Other compounds such as sugars, amino acids and carboxylate anions are transported across the membranes with the help of carrier proteins, their direction of movement is dependent on the electrochemical gradient formed (Rohrbacher & St-Arnaud 2016). Higher molecular weight compounds such as secondary metabolites, polysaccharides and proteins are secreted into the rhizosphere with the aid of membrane bound proteins in an active process at the expense of ATP. The transport protein families so far detected include the multidrug and toxic compound extrusion (MATE), ATP-binding cassette

(ABC), the major facilitator superfamily (MFS) and the aluminium-activated malate transporter (ALMT) (Baetz & Martinoia 2014; Huang *et al.* 2014). This active transport is important because it indicates a specific plant control on the exuded substances.

The root system architecture plays a big role in acquiring resources in the soil environment and it is postulated to have a significant role in determining the composition of exudates (Badri & Vivanco 2009). At the root tips and sites of lateral branching there is an intense activity due to cell division, these are hence the regions that contain the highest concentration of carbon resources. This consequentially develops steep electrochemical gradients that lead to exudation by passive diffusion, making these the sites with highest concentration of exudates (Dennis *et al.* 2010). Exudation on older parts of the roots also occurs though to a lower extent (Badri & Vivanco 2009).

2.2.3 – Bacteria – exudate interactions in the rhizosphere

Overall, plants produce a compositionally diverse set of more than 100000 different compounds that can be secreted into the rhizosphere (Haichar *et al.* 2014), but functional determination in the rhizosphere is restricted to a few of these compounds (Table 1).

Table 1 – Function of exuded compounds from different plant rhizospheres (Haichar *et al.* 2014).

Exudates component	Functions	Specific compounds identified in root exudates
Organic acids	Nutrient source	Citric, glutaric, oxalic, malonic
	Chemoattractant signals to microbes	Malic, aldonic, fumaric, erythronic
	Chelators of poorly soluble mineral nutrients	Succinic, ferulic, acetic, butanoic
	Acidifiers of soil	Butyric, syringic, valeric, rosmarinic, lactic, glycolic
	Detoxifiers of Al	*trans*-cinnamic, piscidic, formic
	nod gene inducers	aconitic, pyruvic
		vanillic, tetronic
Amino acids	Nutrient source	a- and β-alanine proline
	Chelators of poorly soluble mineral nutrients	asparagine, valine, threonine, aspartate, tryptophan cystein,
	Chemoattractant signals to microbes	ornithine, cystine, histidine, glutamate, arginine, glycine, homoserine, isoleucine, phenylalanine, leucine, -Aminobutyric acid, lysine a-Aminoadipic acid, methionine, serine, homoserine
Sugars & Vitamin	Promoters of plant and microbial growth nutrient source	Glucose, desoxyribose, oligosaccharides galactose, biotin, maltose, thiamin, ribose, niacin, xylose, raffinose pantothenate, rhamnose, rhiboflavin, arabinose, fructose
Proteins and enzymes	Catalysts for P release from organic molecules	Acid/alkaline, phosphatase
	Biocatalysts for organic matter transformations	amylase, invertase, protéase
	Plante defence	PR proteins, lipases, β-1,3-glucanases
Purines	Nutrient source	Adénine, guanine, cytidine, uridine
Inorganic ions and gases	Chemoattractant signals to microbes	HCO_3^- OH^- H^+ CO_2 H_2
Phenolics	Nutrient source	Liquiritigenin, luteolin
	Chemoattractant signals to microbes	Daidzein, 4′,7-dihydroxyflavanone
	Microbial growth promoters	Genistein, 4′,7-dihydroxyflavone
	nod gene inducers and inhibitors in rhizobia	Coumetrol, 4,4′-dihydroxy-2′-methoxychalcone
	Resistance inducers against phytoalexins	Eriodictyol, 4′-7-dihydroxyflavone
	Chelators of poorly soluble mineral nutrients	3,5,7,3′-tetrahydroxy- 4′methoxyflavone
	Detoxifiers of Al	naringenin
	Phytoalexins against soil pathogens	isoliquiritigenin, 7,3′-dihydroxy-4′-methoxyflavone
		umbelliferone, (+)- and (−)- catechin
Root border cells	Produce signals that control mitosis	
	Produce signals controlling gene expression	
	Stimulate microbial growth	
	Release chemoattractant	
	Synthesize defense molecules for the rhizosphere	
	Act as decoys that keep root cap infection-free	
	Release mucilage and proteins	

While the roles of primary metabolites (such as amino acids and sugars) as nutrient sources are well elucidated, reports suggest that these also can control gene expression in rhizosphere bacteria independently of trophic effects (Drogue *et al.* 2013). An example is the selection of *Mesorhizobium tianshanense* to the rhizosphere of the liquorice plant, *Glycyrrhiza uralensis,* through the activation of the *msiA* gene by the exuded compound canavanine. This gene encodes a putative translocator that specifically exports canavanine and therefore renders these bacteria immune to the toxic effects of the amino acid analogue (Cai *et al.* 2009). Another study mapped sugar and amino acid content in *Avena barbata* root exudates which highlights the availability of tryptophan on root tips (Jaeger *et al.* 1999). This is of relevance since tryptophan (Trp) is a biosynthetic precursor of indoleacetic acid (IAA), an auxin produced by PGPR that enhances plant growth. Besides a precursor effect, Trp was shown to affect auxin levels produced by *Azospirillum brasilense* and *Enterobacter cloacae*, by modulating the transcription of the *ipdC*/*ppdC* gene which encodes for the indole-3-pyruvate decarboxylase activity involved in IAA biosynthesis (Drogue *et al.* 2013). Apart from the wide range of roles that it serves on plants, γ-aminobutyric acid (GABA) is a common component of root exudates and has been proposed to serve as signalling molecule for the recruitment of bacteria to the rhizosphere. GABA was shown to be used as sole carbon and nitrogen source by *Pseudomonas putida* KT2440 which also upregulates a GABA importer when growing in the rhizosphere of corn seedlings (Moe 2013). Organic acids have also been shown to attract bacteria, such as the tricarboxylic acid cycle intermediate L-malic acid. This compound can recruit the beneficial bacteria *Bacillus subtilis* FB17 in the *Arabidopsis thaliana* rhizosphere, upon plant attack by the foliar pathogen *Pseudomonas syringae* pv *tomato* (Rudrappa *et al.* 2008). In the rhizosphere of tomato, both malic and citric acid are the major chemoattractants for *Pseudomonas fluorescens* WCS365, triggering a chemotactic response (de Weert *et al.* 2002). A recent study has pinpointed that disruption of the catabolism pathway for arabinose and protocatechuate in *Rhizobium leguminosarum* bv *viciae* may affect competitiveness of this bacterium in the pea rhizosphere (Garcia-Fraile *et al.* 2015).

In addition to primary metabolites, roots secrete a large range of secondary metabolites with multiple functions, including the cross kingdom signalling to rhizosphere bacteria. A well-studied example of positive interactions between plants and rhizobacteria is the highly specific relationship between legumes and gram-negative soil rhizobia. Under nitrogen limiting conditions, chemical signals from the legume (mostly flavonoids) activate specific lipo-chitooligosaccharide signaling compounds, called Nod factors, in rhizobia. The perception of specific Nod factors triggers a signalling cascade in the host that leads to the formation of nodules that can be colonized by the bacteria, which have the capacity to fix nitrogen. The chalcone 4, 4'-dihydroxy-2'-methoxychalcone

(methoxychalcone) secreted by *Medicago trunculata* and *Vicia faba* can induce the *nod* genes in *Sinorhizobium meliloti,* and *Rhizobium leguminosarum* bv *viciae* and *R. leguminosarum* bv *trifolii*, respectively (Liu & Murray 2016). *Nod* genes of *Bradyrhizobium japonicum* are induced by the isoflavonoids genistein and daidzein released by soybean (Swamy *et al.* 2016). Several other flavonoids and even non-flavonoid compounds like xanthones have been reported to activate rhizobial *nod* genes (Janczarek *et al.* 2014). In a similar fashion, actinorhizal plants establish a symbiosis with *Actinobacteria* from the genus *Frankia*. Nodulation seems to be influenced by phenolics such as cinnamic, benzoic and hydroxybenzoic acids, and the compounds flavanone and isoflavanone (Haichar *et al.* 2014).

Although the association of nitrogen fixing bacteria with plant roots triggered by flavonoids is the best studied so far, other secondary metabolites were shown to elicit a positive response from rhizosphere bacteria. Benzoxazinoids are present in the root exudates of grasses and are involved in plant defence against pests and diseases aboveground, but some have been reported to also have a role belowground. An example is 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA), a benzoxazinoid that elicits a chemotactic response of the plant beneficial *Pseudomonas putida* KT2440 in maize rhizosphere (Neal *et al.* 2012). Plant roots also secrete compounds that mimic the bacterial cell density dependent quorum-sensing (QS), in order to stimulate or repress bacterial responses. The most common QS system in Gram-negative bacteria uses N-acyl homoserine lactones (AHL) as signals involving two regulatory genes, a member of the *luxI* family of AHL synthase genes and a member of the *luxR* family of AHL-responsive transcriptional regulatory genes (Schaefer *et al.* 2013). It has been recently discovered that plant associated bacteria possess proteins closely related to QS LuxR, that do not respond to AHL but instead to plant low molecular weight compounds (Venturi & Keel 2016). This effect has been demonstrated for *Sinorhizobium fredii* SMH12 and *Pantoea ananatis* AMG501 which have their capacity of establishing biofilms impaired due to AHL-mimic QS molecules from *Oryza sativa* (rice) and *Phaseolus vulgaris* (bean) (Pérez-Montaño *et al.* 2013). Similarly, root exudates from *Arachis hypogaea* L. (peanut) could stimulate root colonization and synthesis of AHLs with long acyl chains by *Bradyrhizobium* strains (Nievas *et al.* 2017). Despite the various reports, the chemical nature of these AHL mimics remains unknown. In addition, proteins in root exudates are possible signaling pathways. This is reflected mostly on arabinogalactan proteins, which are highly glycosylated members of the hydroxyproline-rich glycoprotein superfamily of plant cell wall proteins (Nguema-Ona *et al.* 2013). A study has shown that *Rhizobium leguminosarum* bv *viciae* exhibited a novel mode of arabinogalactan-induced polar attachment in the rhizosphere of peas, other legumes, wheat and *Arabidopsis thaliana* (Xie *et al.* 2012; Swamy *et al.* 2016).

Although evidence is growing regarding the exudation profiles of several plant species, there is only limited knowledge regarding the role of individual compounds in attracting bacteria and this is mostly restricted to one-to-one interactions since multipartite interactions are highly complex. The identification of the compounds present in the root exudates that influence the rhizobacterial community structure and function in natural environments would help to develop strategies for improving plant performance and increasing crop yield and sustainability (Huang *et al.* 2014).

2.2.4 – Implications of rhizosphere engineering for agriculture

Plant microbiome discoveries could advance sustainable agriculture through the manipulation or direction of plant-microbe interactions, with the goal of establishing crops that selectively enhance beneficial functions of their accompanying microbiota. This rhizosphere engineering can be achieved in two ways, by either manipulating the plants or their microbiome (Figure 4).

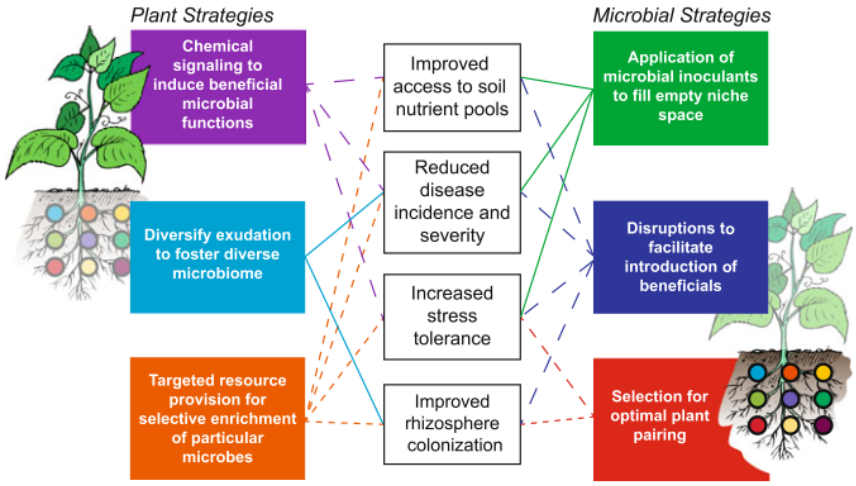

Figure 4 – Possible strategies for rhizosphere engineering (Bakker *et al.* 2012).

Microbiome-based approaches focus on the direct inoculation of beneficial microorganisms either individually or as mixed cultures. Inoculants of PGPR can promote plant growth by stimulating plant hormone production and prompting disease suppression by promoting induced systemic resistance in plants or production of antifungal compounds, antibiotics and bacteriocins (Haldar & Sengupta 2016). An example is the screening of 10,000 soil and plant isolates, leading to the identification of a mix of three *Pseudomonas* strains that decrease the severity of plant disease triggered by pathogens of the genus *Pectobacterium* when inoculated in the potato rhizosphere. Interestingly, each *Pseudomonas* strain used alone exhibited only limited or nonexistent biocontrol activity (Dessaux *et al.* 2016).

The use of microbial inoculants rests on the availability of cultured isolates and so this approach is limited to the small fraction of microorganisms that readily grow in laboratory conditions. Furthermore, research is necessary to evaluate the long-term maintenance of these inoculants, since these microorganisms can be outcompeted in the soil. In an attempt to minimize these problems, recombinant microbial strains are now being engineered, but this also raises concerns, and hence requires a thorough assessment, of the of potential rapid spread of these novel genes by horizontal gene transfer. The first report on engineering was the construction of a plasmid containing the *chiA* gene from *Serratia marcescens*, heterologously expressed in *Escherischia coli*. This gene promotes the degradation of chitin and proved effective in reducing disease caused by the fungi *Sclerotium rolfsii* in bean and *Rhizoctonia solanii* in cotton (Shapira *et al.* 1989). More recently, drought tolerance and yield in common bean (*Phaseolus vulgaris*) was improved by overexpressing trehalose-6-phosphate synthase in the symbiotic bacterium *Rhizobium etli*. This led to plants with more symbiotic nodules with increased nitrogenase activity and higher biomass (Suárez *et al.* 2008). In order to enhance the success of new microbial inoculants in the soil, new approaches are employed. This is, namely, the introduction of disturbances with the goal of increasing rhizosphere vulnerability to invasion by the applied microorganisms, through application of fungicides, crop rotation or tilling (Bakker *et al.* 2012).

A recent view on rhizosphere engineering looks on the holobiont concept, taking into account the metaorganism and trying to optimize the whole system instead of the partners separately (Quiza *et al.* 2015). This approach can be made effective through crop rotation and inoculation of genetically modified plants with genetically modified microorganisms (Haldar & Sengupta 2016). Crop rotation is an effective way to amplify the spectrum of diversity and ecological services to crops. This involves culturing of plants in turns, so that the beneficial microorganisms and phytochemicals in the soil from one plant might help the next, and thereby establishing a synergy between cultures. This increased plant diversity leads also to a more diverse microbial community which enhances the resilience against pathogens (Quiza *et al.* 2015). Although promising, these new approaches are still very specific, focusing on one trait or interaction and disregarding the consequences for the entire microbial community.

2.3 – Microbial community assembly

One of the biggest and most difficult goals in microbial ecology is the understanding of the processes that govern the observed patterns of species abundances and functions over space and time. Several of these patterns have been observed for microbial communities, like species abundance

distributions, species-area, distance-decay, and species-time relationships but there is controversy regarding the controlling mechanisms (Vellend 2010; Nemergut *et al.* 2013).

2.3.1 – Ecological processes

Recently a conceptual framework has proposed the ecological processes of selection, diversification, dispersal and drift as responsible for community assembly (Nemergut *et al.* 2013).

Traditional niche-based theory gives much emphasis to deterministic factors (species traits, interspecies interactions and environmental factors) as governing microbial community structure. On the opposite, neutral theory assumes that all individuals are functionally equivalent and therefore community structure is governed by the stochastic processes of birth, death, colonization, extinction and speciation. Nevertheless it is becoming more and more evident that both processes are important for the assembly of microbial communities (Zhou & Ning 2017) (Figure 5).

Figure 5 – Deterministic versus stochastic processes. The widths of the blue and orange parts represent the importance of determinism and stochasticity associated with ecological processes (Zhou & Ning 2017) (adapted).

These theories arise from macroecology and more recently have been employed to microbial ecology, though there are several particularities of microorganisms that need to be considered. Though microorganisms do not actively disperse, passive dispersal has much more impact on their distribution than for macroorganisms, mainly due to the small size and rapid transport of microbes. Another very important difference is the fact that microorganisms can enter a reversible state of dormancy depending on environmental stressors. Also, microorganisms have short generation times and can readily exchange genetic material, all of which leads to rapid evolution (Nemergut *et al.* 2013).

2.3.1.1 – Selection

Selection is a major force shaping bacterial communities and results from biotic and abiotic pressures which cause variation in reproductive success across individuals and species (Stegen *et al.*

2013). It is therefore a deterministic process. As mentioned before, there is an ample array of data showing that abiotic factors such as pH and temperature determine the structure of bacterial communities. Less studied, but also important is the role of biotic interactions such as parasitism and commensalism (Zhou & Ning 2017). When environmental conditions (biotic or abiotic) are homogeneous, the selective pressure is constant and therefore there is little variation on the community composition. This is referred to as homogenous selection. On the other hand, when environmental conditions are variable through space and time there is a shift in selective pressure, possibly leading to high compositional turnover. This is referred to as variable or heterogeneous selection (Stegen *et al.* 2015).

2.3.1.2 – Dispersal

Organismal dispersal refers to the movement of organisms and their successful establishment in space. Its influence on community dynamics depends on the size and composition of communities where the organisms come from and where they arrive. Continuously high rates of dispersal have the potential to overwhelm the effects of selection and therefore enforce a low community turnover over time, in a process referred to as homogenizing dispersal. On the other hand, low levels of dispersal constrain the exchange of organisms among communities, which can result in spatial turnover in composition, in a process referred to as dispersal limitation. Dispersal is a very important process in case of primary succession, where it is required for community establishment and for an increase in diversity (Vellend 2010; Stegen *et al.* 2013, 2015).

Dispersal is a poorly understood process in the bacterial world, which is due to small size of microbes, their wide geographic distribution and short generation times. It is still an open question in microbial ecology whether bacteria are dispersal limited or not, but some studies show biogeographical patterns which points to at least some degree of dispersal limitation (Cho & Tiedje 2000; Whitaker *et al.* 2003). Dispersal is viewed as a passive process for microorganisms, occurring via water or air transport, or when there is an association with higher organisms that actively disperse (Nemergut *et al.* 2013). This implies that dispersal is a neutral process, and this holds true if one considers only population size, where more abundant species have a bigger probability to disperse. However, different species have distinct traits that can enhance or decrease their ability to disperse (such as spore formation). Moreover, the newly arrived bacteria are subject to environmental conditions of the new habitat which will determine their survival and they may experience priority effects (the species that arrives early exhausts or modifies available resources, and by that affects the establishment success of later arriving species (Svoboda *et al.* 2017)). Therefore dispersal is not a purely stochastic process (Zhou & Ning 2017).

2.3.1.3 – Diversification

Evolutionary diversification is the process of generating new genetic variation, which is crucial for the understanding of the origin and maintenance of biodiversity. Diversification is mostly overlooked in ecology: for macroecology it involves long-term evolutionary processes of millions of years and in microbial ecology, it is difficult to study empirically. Nevertheless, this process is important for explaining bacterial community structure, especially because it occurs at much faster rate in microorganisms due to their short generation times (Zhou & Ning 2017). In the case of bacteria, diversification is mostly driven by mutation and horizontal gene transfer, which are mostly random events. This makes diversification a stochastic process, although in some aspects, it can also be deterministic. One example is the ability of some bacteria to undergo dormancy periods in which no evolutionary processes take place (Nemergut *et al.* 2013). Also, different bacteria are found to have distinct mutation rates and therefore can evolve at different paces.

2.3.1.4 – Drift

Ecological drift involves stochastic changes in relative abundances of species in a community over time as a result of the random processes of birth, death and reproduction. The importance of this process is highest in small and isolated populations, such as host-associated communities, due to the increased importance of sampling effects (when communities are assembled at random from a pool of species, more diverse mixtures have a higher probability of containing a species with extreme traits which may drive some ecosystem processes if it becomes dominant) (Hector *et al.* 2002; Gilbert & Levine 2017). Also, rare species in a community are the most susceptible to drift, since even slight negative changes in their abundance can lead them to extinction (Zhou *et al.* 2017). Ecological drift has only a strong effect on communities if selection is weak since strong selection will override any stochastic effects of drift. Nevertheless, differences in competitive ability drive inferior species to low abundance, where demographic stochasticity plays a strong role (Chase & Myers 2011; Gilbert & Levine 2017).

2.3.2 – Ecological succession

Ecological succession relates to the change in species composition and structure over time either on newly created surfaces with little or no biological legacy (primary succession) or following disturbance events where some biological legacy remains (secondary succession) (Walker *et al.* 2010; Walker & del Moral 2011). The development of soil and vegetative gradients is defined by an

interactive set of dynamic feedbacks fundamental for the process of succession and ecosystem development. Although succession of plant communities has been studied for a long time, little is known about belowground microbial communities' relationship to this process (Cui *et al.* 2012; Jangid *et al.* 2013).

2.3.2.1 – Bacterial primary succession in soil

The development of bacterial communities during soil primary succession has been evaluated along receding glaciers (Sigler *et al.* 2002; Nemergut *et al.* 2007; Knelman *et al.* 2012; Brown & Jumpponen 2014; Schmidt *et al.* 2014; Castle *et al.* 2016), former lava fields (Cutler *et al.* 2014; Kelly *et al.* 2014) and rhizosphere of different plants (Chaparro *et al.* 2014; Shi *et al.* 2015; Tkacz *et al.* 2015).

The trajectories of plant communities undergoing primary succession can vary, but for bacterial communities the question remains as to whether communities in different sites follow the same successional trajectories through ecosystem development (Castle *et al.* 2016). A recent study assessed bacterial community structure changes associated with the long-term ecosystem development (60 to c. 120000 years) of the Franz Josef chronosequence in New Zealand. Results indicate that variation in the communities is greatest during the earliest years and slows down with ecosystem development, being consistent with pedogenesis and vegetative succession (Jangid *et al.* 2013). A previous study in the same chronosequence identified higher fungal to bacterial ratios in older sites, which were related to a decline in phosphorous availability (Allison *et al.* 2007), which suggests a bigger bacterial role at early stages of ecosystem development. Different strategies of community development between bacteria and fungi were also suggested in a study evaluating soil development in a chronosequence of the Lyman glacier, 0 to 70 years since deglaciation. This work emphasizes the importance of microbial early primary succession for the development of a pool of organic carbon and nitrogen in the soil prior to plant establishment. Bacterial communities were more affected by distance and vegetation cover than fungal communities and converged to a community type along the chronosequence, whereas fungal community assembly appeared more stochastic (Brown & Jumpponen 2014; Schmidt *et al.* 2014). Distinct results were observed on an 850-year chronosequence of lava flows in Iceland, where fungal community structure paralleled changes in aboveground vegetation, monotonically increased in diversity with increasing terrain age, while bacterial communities did not vary across the chronosequence. This stability could be linked to the soil properties (pH and soil organic matter) which also remained constant (Cutler *et al.* 2014).

2.3.2.2 – Artificial soils

The highly complex nature of soils makes a deep understanding of ecological processes difficult. Therefore, more simplistic alternative approaches have been exploited, such as the use of artificial soils. These are mixtures of defined organic and mineral compounds that mimic the natural soil composition and thereby allow the importance of specific components to be analysed (Ellis 2004; Hemkemeyer *et al.* 2014). Artificial soils have been used for several years under laboratory conditions, focusing on pedogenesis and organic matter formation in the early stages of soil development (Weigand & Totsche 1998; Leifeld *et al.* 2001; Heckman *et al.* 2013; Kallenbach *et al.* 2016). Despite allowing studies of early-stage soil primary succession in a controlled environment, artificial soils have rarely been used to study microbial colonisation patterns (Ding *et al.* 2013; Babin *et al.* 2014; Hemkemeyer *et al.* 2014; Ditterich *et al.* 2016).

2.4 – Culture-independent approaches to study soil bacterial communities

The large majority of bacteria which exist in nature have not yet been cultured in the laboratory. Therefore, the primary source of information for these organisms are their macromolecules, such as nucleic acids, lipids and proteins. Since the 1980s, the application of molecular ecological methods, especially those based on surveys of genes after polymerase chain reaction (PCR) amplification, has allowed cultivation-independent studies of the microbial communities in diverse environments (Su *et al.* 2012) (Figure 6). Distinct methods offer different information and can be sorted by their ability to profile microbial biomass, diversity or activity (Rincon-Florez *et al.* 2013). Often these methods are used in combination in order to have a broader insight into soil bacterial communities.

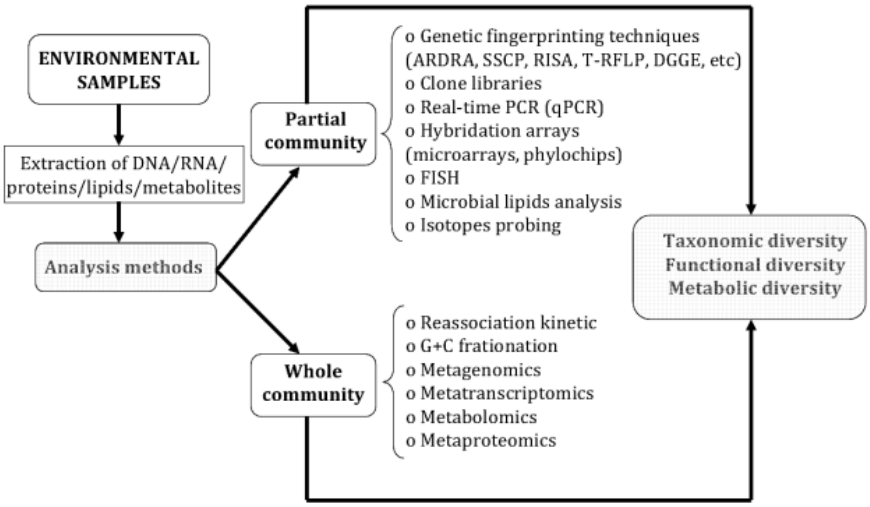

Figure 6 – Culture-independent methods to characterize microbial community diversity in the environment (Escalante *et al.* 2014).

2.4.1 – Assessing biomass

Biomass measurements are important to characterize soil properties and to facilitate linking changes in microbial community structure to changes in process performance. Distinct methods which allow to quantitatively evaluate a microbial population have been developed. One of the most commonly used is the chloroform fumigation extraction (CFE) method (Setia *et al.* 2012), where soils are exposed to chloroform vapour to lyse the cells and then the components are extracted. The C, N and P difference between fumigated vs. non-fumigated soils is the chloroform-labile pool and it is proportional to microbial biomass C, N or P (Jenkinson *et al.* 2004). In a recent study, microbial biomass (C, N and P) and microbial community composition were analysed in order to study the influence of microbial mineralization and immobilization of nutrients on soil fertility. Microbial biomass stoichiometry in P-poor soils was more susceptible to additions of C, N and P than in the P-rich soils, and even in P-poor soils microorganisms were C-limited (Heuck *et al.* 2015).

Phospholipids are derived from cellular membranes and have different chain lengths and composition, which can be used to distinguish specific microbial taxa (Rincon-Florez *et al.* 2013). These are extracted from viable cells since they rapidly degrade upon death. The membranes of bacteria and eukaryotic cells contain phospholipid fatty acids (PLFAs), while archaeal cell membranes contain phospholipid etherlipids (PLELs) and fungi contain ergosterol (Hirsch *et al.* 2010). Recently, the effect of agricultural land use change on soil biomass was studied for afforested, cropland and uncultivated soils in central China. Afforestation averaged higher microbial PLFA biomass when compared with cropland and uncultivated soils, with higher values in top soils than deep soils. Furthermore, microbial PLFA biomass was strongly correlated with soil organic nitrogen and labile carbon (Zhang *et al.* 2016).

With the development of molecular biology and verified specific target genes, the quantitative PCR (qPCR) became a powerful technique to measure the total or specific microbial absolute abundance in soil (Lou *et al.* 2018). qPCR employs fluorescent labelled probes and dyes to measure the progression of DNA amplification during the reaction (Maddocks & Jenkins 2017). It is also possible to measure RNA with reverse transcriptase Q-PCR (RT-PCR) (Bustin *et al.* 2005). This is a frequently used technique, employed mainly in combination with sequencing for the study of bacterial community structure in different soil habitats (Navarrete *et al.* 2013; Maestre *et al.* 2015; Siles & Margesin 2016).

2.4.2 – Assessing diversity

The diversity and composition of soil bacterial communities is thought to have a direct influence on ecosystem processes. Some of the first developed are fingerprinting methods, which are based in PCR amplification of fragments by selected primers. These can be divided into two groups according to the differential electrophoretic migration on gels: (i) size-dependent migration (such as terminal restriction fragment length polymorphism (T-RFLP), automated ribosomal intergenic spacer analysis (ARISA/RISA), random amplified polymorphic DNA (RAPD) and single-strand conformation polymorphism (SSCP)) and (ii) sequence-dependent migration (such as denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE)) (Rincon-Florez *et al.* 2013). Fingerprinting methods generally only reveal fractions of the microbial communities, and due to the progress in next generation sequencing, which allows a more comprehensive analysis, fall into disuse.

Metagenomic approaches are now the most widely used to estimate the structure and functional potential of microorganisms found in soil samples. Most soil metagenomic studies rely on taxonomic or functional annotation of short reads based on curated databases (Delmont *et al.* 2015). The 16S rRNA gene sequence became the most widely used marker gene for profiling bacterial communities, since it contains both highly conserved regions for primer design and hypervariable regions to identify phylogenetic characteristics of bacteria (Tringe & Hugenholtz 2008). Large-scale coordinated efforts to explore soil taxonomic and functional diversity are now in place, such as the Earth Microbiome Project (Thompson *et al.* 2017), the Ecological Function and Biodiversity Indicators in European Soils (EcoFINDERS; (http://ecofinders.dmu.dk/) and MicroBlitz (http://www.microblitz.com.au/). The abundance of sequencing studies enabled a recent large study addressing macroecological questions of bacterial diversity, where thirty independent high throughput sequencing datasets, comprising 1,998 soil samples from 21 different countries, were combined. Researchers found rare taxa to be more important for structuring soil communities than abundant taxa and better predictors of community structure than environmental factors (Ramirez *et al.* 2018). Despite informative, these studies rely mostly on amplicon sequencing of various hypervariable regions of the 16S rRNA gene, and the limited sensitivity of short reads restrains the fuller explanation of the available sequencing information. An alternative is to assemble short reads back together to reconstruct genomes and other genetic structures in a *de novo* manner (Delmont *et al.* 2015). Although there are many challenges to overcome when dealing with massive quantities of sequence information and assembling the short reads (Howe *et al.* 2014), this approach has revealed the potential ecological roles of important soil bacteria such as the verrucomicrobial member '*Candidatus* Udaeobacter copiosus' (Brewer *et al.* 2016). Third-generation sequencing has recently

become available, allowing direct sequencing of more than 10,000 bp reads. This holds promise for future diversity studies, enabling much more information to be obtained. Nevertheless, high error rates and cost prevent its routine use (Liu *et al.* 2017).

Fluorescence *in situ* hybridization (FISH) enables in situ phylogenetic identification and enumeration of individual microbial cells by whole cell hybridization with oligonucleotide probes. Due to problems with weak signal intensities and high background autofluorescence in environmental samples, catalysed reporter deposition (CARD) was successfully combined with FISH (CARD-FISH) and the resulting enhancement of signal intensity improved the detection and quantification of single cells in various soil environments (Schmidt & Eickhorst 2014). This method has helped to unravel the spatial distribution of bacteria associated with plants (Bulgarelli *et al.* 2012; Bao *et al.* 2014; Schmidt & Eickhorst 2014) and in different soil environments (Li *et al.* 2015; Probandt *et al.* 2018).

2.4.3 – Assessing activity

Soil microbial biomass, biodiversity and even DNA-based metagenomics approaches may not provide a direct measure of microbial activities in the soil. Microbial activity profiles based on actual metabolic activity are therefore more likely to reveal important microbial ecosystem functions (Rincon-Florez *et al.* 2013). The techniques more frequently employed are stable isotope probing (SIP) and RNA-based next generation sequencing (metatranscriptomics).

Stable isotope probing (SIP) can physically link the fluxes of elements to the genome of an organism. An environmental soil sample is mixed with a precursor (such as sugars or aminoacids) that has been labelled with a stable heavy isotope such as ^{15}N or ^{13}C, (Hungate *et al.* 2015). If the organisms in the sample metabolize the substrate provided, they incorporate the heavy isotope into their nucleic acids, which will be "heavier" and can be separated from the non-labelled ones by centrifugation. The nucleic acids obtained can then be used directly for next-generation sequencing (Clark & Pazdernik 2016). This method has been employed to identify bacteria assimilating plant derived carbon from different plants, such as *Arabidopsis thaliana* (Haichar *et al.* 2012), potato (Rasche *et al.* 2009) and rice (Hernández *et al.* 2015).

Metatranscriptomics is the study of rRNA and mRNA of a microbial community in an environment through sequencing, allowing the simultaneous investigation of the gene expression (mRNA) and abundance (rRNA). This is one of the most powerful tools for understanding the timing and regulation of complex microbial processes within communities and microbial responses to changing conditions (Moran 2009). As metatranscriptomics involves direct sequencing of reverse-transcribed RNA (RNAseq) and requires no a priori knowledge of the sequences present, it is also proving useful

as a tool to detect novel microbial small RNAs (Shi *et al.* 2009). This method has provided new and important knowledge about soil ecosystems (Urich *et al.* 2008; Shrestha *et al.* 2009; Tveit *et al.* 2014). A recent study analysing an agricultural soil with a long history of usage of chemical fertilizers and pesticides revealed several important bacteria involved in pesticide degradation and heavy metal detoxification. Furthermore, the detailed analysis of various metabolism revealed that bacteria in this ecosystem are dependent on organosulfonated compounds for their growth and development (Sharma & Sharma 2018).

2.5 – Culture-dependent approaches to study soil bacterial communities

Currently, access to uncultured bacteria relies on genome sequencing, metagenomics and single-cell genomics; tools which give insight into the organisms physiological capacities. Nevertheless, only the isolation and characterization of these as-yet-uncultured bacteria can lead to the actual comprehension of their detailed metabolism, functions and ecological role (Overmann 2013).

The observation that not all bacteria will readily grow in laboratory conditions was made long ago, when microscopic observations revealed much higher bacterial cell numbers than the number of colonies able to grow in a Petri dish. This discrepancy was referred to as the "great plate count anomaly" and holds true still today (Stewart 2012). Over the past few decades the number of small subunit ribosomal RNA gene (SSU rRNA) sequences deposited in databases is increasing exponentially, revealing that much of the bacterial diversity remains elusive to cultivation attempts. Total prokaryotic diversity has been estimated between 10^7 to 10^9 species, but from these only 0.1% to 0.001% have been recovered through diverse cultivation attempts (Overmann 2013). Furthermore, cultivation is highly biased: all bacteria that are indeed culturable are affiliated with only 30 out of ~80 recognized bacterial phyla, with 90% of these species falling into only the four phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* (Overmann *et al.* 2017) (Figure 7).

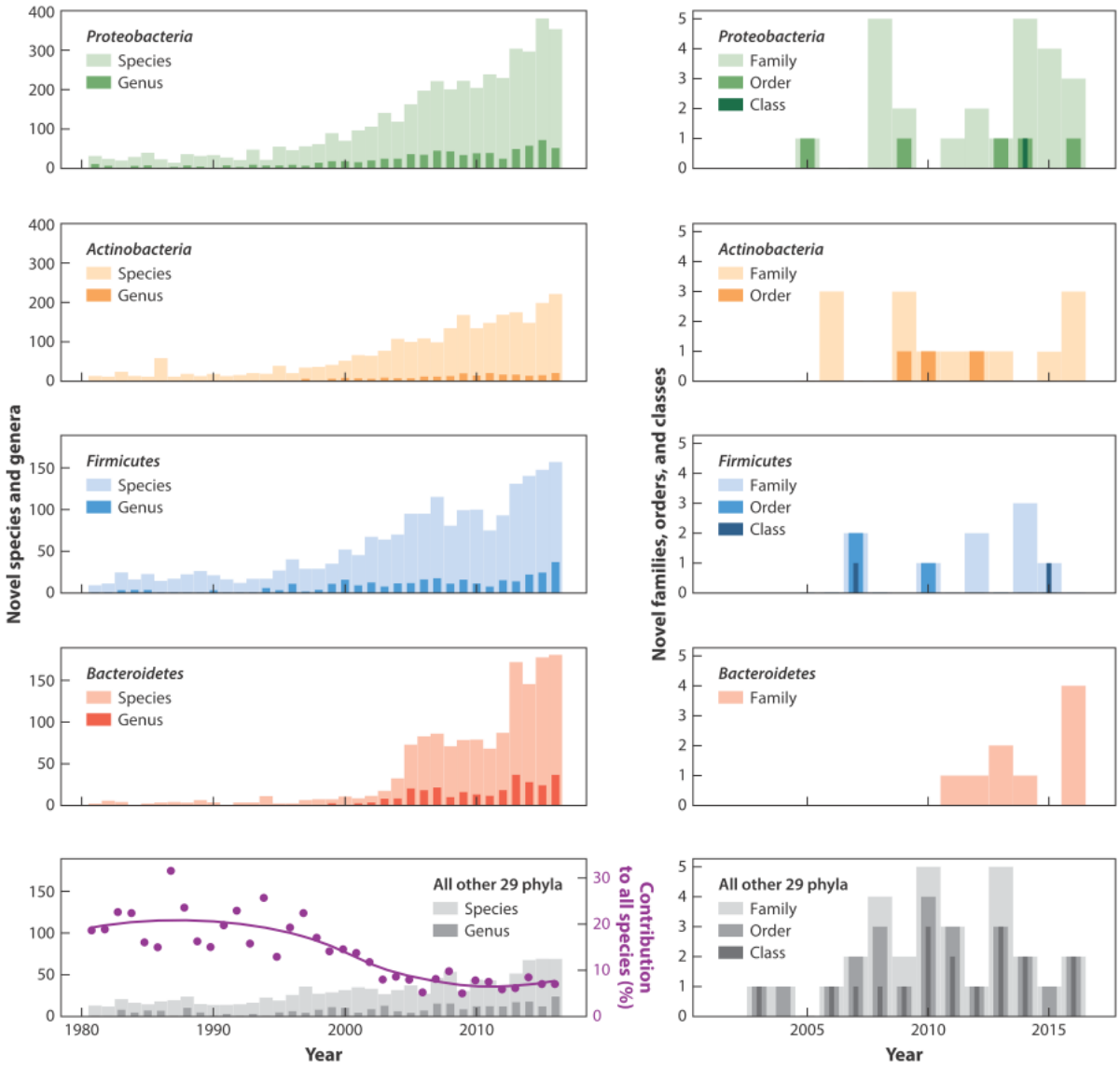

Figure 7 – Affiliation of bacterial taxa described in the recent decades and their novelty (Overmann *et al.* 2017).

2.5.1 – Reasons for low cultivation success

Branding an organism "not-yet-cultivated" does not reflect an inherent inability of being unable to grow in the laboratory, but rather describes a severe lack of knowledge regarding the organism biology. It is obvious that unculturable bacteria are alive and replicating in their natural environments, and it is evident that for the great majority of the cases we fail to replicate these the critical conditions *in vitro* (Stewart 2012).

2.5.1.1 – Physiological state and adaptation of bacteria to the heterogeneous environment

Most of all bacterial strains isolated to date have fast growing capacities and are adapted to a range of substrates, but these characteristics do not seem to correspond to the characteristics of most bacteria in an environment like soil. Cellular ribosome content of bacteria in soils was found to be three orders of magnitude lower than of exponentially growing bacterial cultures, which indicates that bacteria in soil are subject to pronounced starvation due to the limitation of carbon compounds (Overmann *et al.* 2017). To survive extended periods of adverse conditions such as starvation, many bacteria switch to a metabolically inactive, dormant state. These cells are often not capable of resuming growth in nutritionally rich medium, possibly due to an imbalance in metabolism producing a near instantaneous production of superoxide and free radicals (Bloomfield *et al.* 1998), requiring instead media with a reduced carbon content (Azevedo *et al.* 2012). Many bacteria in their natural environments are thought to be slow growing, with estimated generation times of soil bacteria between 120 and 180 days (Hobbie & Hobbie 2013). This fastidious nature also hampers their cultivation, as some bacteria need several months or even years to develop in pure culture (Davis *et al.* 2011; Huber *et al.* 2014).

Bacterial adhesion to particles is an ubiquitous process in soil, as concluded from microscopic observations, from the lack of movement of surface bacteria to underlying soil layers and groundwater during heavy rains, floods or irrigation, and from the increased recovery of bacteria from soil with the use of sonication or surfactants (Chenu & Stotzky 2002). This indicates that some bacteria are specifically adapted to life in a biofilm, requiring a suitable surface for growth. This characteristic has already been exploited for cultivation purposes, and it was demonstrated that bacterial biofilms on different surfaces are enriched with previously unculturable bacteria (Gich *et al.* 2012).

Motile bacteria have developed mechanisms to detect suitable growth substrates and can therefore move towards them. This chemotactic ability is very useful in environments where substrates vary in time, and though it is more pronounced in aquatic environments, it is also a characteristic of some soil bacteria (Scharf *et al.* 2016). So far, this feature has been hardly exploited so far for cultivation purposes due to the difficulty in identifying suitable substrates and concentrations for unculturable bacteria but should be considered for future attempts.

2.5.1.2 – Adaptation to oligotrophy

Many soils are nutrient limited, yet many bacteria live and prosper in this environment. While all bacteria can survive periods in a low-nutrient settings, oligotrophic bacteria are adapted to these and may in fact be incapable of survival in richer environments (Koch 2001). Oligotrophic bacteria are generally characterized by high-affinity uptake systems, low growth rates which are independent of substrate concentrations, small cells, lack of motility and reduced genome sizes (Overmann *et al.* 2017). Members of the phylum *Acidobacteria* have been suggested to be oligotrophs (Fierer *et al.* 2007) and many could indeed be isolated and maintained on media supplemented with comparatively low concentrations of complex carbon sources like yeast extract and peptone (Foesel *et al.* 2013; Huber *et al.* 2014, 2016; Pascual *et al.* 2015; Vieira *et al.* 2017). Similarly, all the validly described bacterial isolates belonging to the *Armatimonadetes* phylum (former OP10) are found to prefer growth in low nutrient media, and in fact are sensitive to high quantities of carbon and nitrogen sources. For cultivation purposes it is therefore important to provide specially formulated media and incubation conditions that consider oligotrophy if one wishes to obtain in pure culture a big fraction of all soil bacteria.

2.5.1.3 – Bacterial interactions

Bacteria are social organisms that establish complex intra- and interspecific interactions. In soils, bacteria occur in close proximity to each other, with distances between cells ranging between 5 and 29 µm, enabling rapid diffusion and an efficient exchange of signalling molecules (Overmann *et al.* 2017). An individual compound produced by one bacteria can have a multitude of functions regarding distinct competitors. One example is bacillaene, a translation inhibitor produced by *Bacillus subtilis* that inhibits the growth of *Streptomyces avermitilis*, interferes with production of pigmented prodigiosin by *Streptomyces coelicolor* and *Streptomyces lividans*, and is involved in defense against consumption by *Myxococcus xanthus* and linearmycin-induced lysis by *Streptomyces* sp. strain Mg1 (Stubbendieck *et al.* 2016). Furthermore, bacteria can establish close symbiotic relationships with higher organisms or (rarely) other prokaryotes, often losing the ability of living outside of the symbiosis, as are the cases of the well known the root nodule symbiosis of rhizobia with legumes or the bacterial symbionts of earthworms which benefit host reproduction while living off the waste products of the host (Lund *et al.* 2014). Co-cultivating bacteria alongside their hosts is an obvious approach to target these bacteria. In the case of bacteria – bacteria interactions, co-cultivation is possible using incubation devices which permit the growth of two different prokaryotes separated by a membrane permeable to metabolites or encasing cells in macromolecular gel

droplets (Zengler *et al.* 2002). Another possible approach is to cultivate bacteria in their natural settings, so they are exposed to the whole array of molecules they might need to grow. Methods include incubation of bacteria on membrane filters and dialysis units, diffusion chambers, agar beads, and Gelrite plugs (Overmann 2013). Recently one of these methods, the iChip, was used for isolation and screening of novel bacteria for secondary metabolite production. This device is comprised of many channels sandwiched between two semi-permeable membranes where environmental samples are diluted and loaded into, which is then buried in soil for incubation. This method led to isolation of a previously uncultured *Betaproteobacteria* (provisionally named *Eleftheria terrae*) which produces the novel antibiotic teixobactin (Ling *et al.* 2015).

Accounting for this cell-to-cell communication in cultivation will be crucial to obtain certain bacteria, but this remains a challenge due to our inability in identifying and adequately reproducing the appropriate conditions. Nevertheless, efforts are underway to pinpoint specific bacterial interactions, mainly though investigation of co-cultures obtained using the dilution-to-extinction method. In a recent study, 115 putative interactions (56 negative and 59 positive) were observed in a high-throughput enrichment cultivation of groundwater samples (Justice *et al.* 2017).

2.6 – Aims of the study

The importance of bacteria for biogeochemical processes in soil hotspots has long been acknowledged. Nevertheless, the available information on the functional role of these bacterial communities at high taxonomic resolution and evidence regarding their establishment and temporal dynamics is still scarce.

Understanding bacterial functions and interactions in plant rhizospheres is of particular importance, due to their impact on plant function and subsequently in ecosystem maintenance. There are several reports elucidating rhizosphere interactions, but much of the research addresses a small range of environmental conditions and subjects plants to artificial settings, often excluding the accompanying community. This approach is clearly limited as the removal of the organisms from their natural context will affect their response. With the recent advances in high throughput sequencing, a comprehensive analysis of the composition of the bacterial communities in the rhizosphere of different plant species (mainly crops) and its links to biotic and abiotic factors has become possible. Evidence is also growing regarding the exudation profiles of several plant species. Nevertheless, very few studies have attempted to analyse the specific plant-bacteria interactions at the community level and to pinpoint the specific signals that drive these associations. In this study we used high-throughput Illumina sequencing to investigate the rhizosphere bacterial community of six plant species *Dactylis glomerata*, *Arrenatherum elatius*, *Alopecurus pratensis*, *Plantago lanceolata*, *Achillea millefolium* and *Ranunculus acris*, which occur naturally in German grasslands. The aim was to identify the major drivers of rhizobacterial community assembly amongst the different plant species across the eight different soil types evaluated. Moreover, we used GC-MS analysis of the root exudate profiles of the six plant species to assess their contribution to the development of distinct rhizobacteriomes.

Identifying the mechanisms and trajectories of soil bacterial communities through ecosystem development and under distinct conditions will improve our understanding of community stability and resilience. Despite numerous previous investigations, it is not clear as to whether bacterial communities in different sites follow the same successional trajectories. Most of the work regarding temporal succession of soil bacterial communities has been focused on secondary succession, following various disturbances. Soil bacteria undergoing primary succession have not received as much attention, though there are many reports of studies in chronosequences, assuming space-for-time substitution. Direct evaluation of the bacterial temporal dynamics has been attempted with the use of artificial soils, but these have so far not been employed in natural settings and therefore do not reflect the actual conditions which bacteria experience in fields worldwide. Hence, high

throughput Illumina sequencing was employed to investigate the surface colonization of artificial minerals newly introduced into natural grassland fields of the same soil type. The goal was to dissect the impact of distinct organic carbon sources (simple versus complex carbon compounds) in the development of distinct communities and evaluate the temporal dynamics of bacteria at a high resolution.

A third goal of this work to employ diverse cultivation techniques in order to retrieve representatives of important players in these grassland ecosystems. The focus of this approach is on the retrieval of oligotrophic bacteria since they are not thoroughly explored. Although they probably constitute a big fraction of soil bacteria and are more adapted to nutrient shortages and other stressors, thy are difficulty to obtain under laboratory conditions, which impairs the assessment of their true role in soil. The bacterial isolates obtained needed to be assessed for their importance in the grassland plant rhizospheres and for the colonization of mineral surfaces. In case of retrieval of novel bacteria, they had to be subject to detailed chemotaxonomic and genomic characterization in order to evaluate their ecological role in soil.

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Chapter 3 – Experimental procedures

This study was accomplished in the framework of the Biodiversity Exploratories, which are large-scale, long-term research sites established in three different regions in Germany: the biosphere reserve Schorfheide-Chorin (Brandenburg, north-eastern Germany), the national park Heinich and its surroundings in Thuringia (central Germany), and the biosphere reserve Swabian Alb in Baden-Wuettemberg (south-western Germany) (Fischer *et al.* 2010).

3.1 – Rhizosphere

Based on their natural abundance in the exploratory grasslands, *Plantago lanceolata*, *Achillea millefolium* and *Ranunculus acris* were selected as representative herb and *Dactylis glomerata*, *Arrhenatherum elatius* and *Alopecurus pratensis* as grass species, respectively. Plant growth, transplantation and collection were done by the Geobotany group of the Institute of Biology / Geobotany and Botanical Garden from the Martin Luther University Halle-Wittenberg (for raising conditions, planting process and experimental setup see Herz *et al.*, 2017). Briefly, plant phytometers were grown axenically in a sterile sand/humus mixture in a greenhouse and were transplanted to the study sites in May to early June 2014. For each of the 3 exploratories, 18 plots were selected (although at the time of sampling, 3 and 1 plots were not accessible in Schorfheide-Chorin and Heinich, respectively, and therefore were skipped) with different soil characteristics and land use regimes.

3.1.1 – Sampling

From the 15th of June to the 21st of July 2015 the plants were excavated and sampled. Soil was removed from the roots and this consisted the bulk soil sample. The plant roots with attached soil (few millimetres around the root) were thoroughly washed in a sterile 0.5% NaCl solution, with vigorous shaking. The root washings were decanted, and the bigger particles recovered; the supernatant was then filtered through 47 mm 0.2 µm polycarbonate filters. Both decanted particles and filters were combined and represented the rhizosphere samples. All material was flash frozen in the field and transported in dry ice. Long term storage was done in liquid nitrogen.

This yielded a total of 219 phytometer plant rhizosphere and 42 bulk soil samples. The rhizosphere of 82 natural occurring individuals of *Plantago lanceolata* and *Dactylis glomerata* were also sampled for the direct comparison with the rhizosphere community present under natural conditions and as reference for the development of the rhizosphere community on phytometer plants.

3.1.2 – Plant root exudates

The information on root exudates was provided by the Metabolomics group of the Department of Stress and Developmental Biology of the Leibniz Institute of Plant Biochemistry. Therefore, they conducted the collection and processing of root exudates, explained in points 3.1.2.1 and 3.1.2.2.

3.1.2.1 – Collection

After collection of the rhizosphere, plant roots were washed and the root exudates were collected following an adapted procedure from Aulakh *et al.*, 2001 (Herz *et al.*, 2018). A second wash step with deionised water was performed to reduce the content of ions from the tap water. After, roots were placed in deionised water of HPLC quality for 2 hours exudation, and the solution was subsequently frozen and stored at -20°C. Water samples without exudation were used control and treated like the exudate samples. Metabolites were extracted of the solutions using a rotary evaporator at 40°C water temperature and 20 mbar pressure and were subsequently dissolved two times in 100% methanol. The change to solvent of measurement was obtained by concentration with a vacuum concentrator and dissolving in 80% methanol including 10 µM ribitol as internal standard. Extracted metabolites were precipitated by centrifugation. The supernatant was dried in a vacuum concentrator and derivatized by methylation with 50µl methoxylamin-hydrochloride (20 mg/ml in pyridine, Sigma Aldrich) for 90 min and silaging with 50 µl BSTFA (Macherey–Nagel) with added alkane retention time indices (C12, C15, C19, C22, C28 (each 0.1 mg ml^{-1} final concentration; Sigma Aldrich) and C32 (0.4 mg ml^{-1} final concentration); Sigma Aldrich)) for 30 min. Both were confirmed at 37 °C.

3.1.2.2 – Gas Chromatography / Mass Spectrometry (GC/MS)

Derivatized exudates and controls were analysed by non-targeted plant metabolite profiling with gas chromatography coupled mass spectrometry (Herz *et al*., 2018).

The measurements were performed by gas chromatograph (6890N GC; Agilent Technologies) equipped with a ZB-5 Zebron Guardian^{TM} Capillary GC column (30 m + 10 m Zebron^{TM}, iD 0.25 mm, df 0.25 µm; Phenomenex) and coupled to Mass Spectrometer (5975 MSD; Agilent Technologies). Samples (2 µL) were injected automatically by Multipurpose sampler (MPS 2XL; Gerstel) in front inlet at 230°C and separated chromatographically with 1 mL/min flow and the following oven program: 1 min 70°C, ramp with 7°C per minute up to 300°C, 5 min 300°C. Transfer line temperature was set at 230°C and ion source at 220 °C. Mass spectra were recorded with 20 Hz. Single samples were derivatized and measured separately with intervals of at least one day. A blank to check for potential

carryover of metabolites during measurement was interspersed every five to six samples. Chromatographic performance and sensitivity was checked by tune evaluation with PSTFA. The raw data were converted to cdf-files by Data Analysis software (Agilent Technologies) and uploaded to the MeltDB software (Neuweger *et al.* 2008). Peak detection with SN = 5 and FWHM = 6 by 315 warped-algorithm and metabolite profiling with threshold = 0.75 for compound conformation was done automatically by MeltDB software. Identification of metabolites by mass spectra similarity was performed automatically by MeltDB based on spectral and index libraries. Gaps in metabolite annotation were manually annotated with the help of MeltDB, sprectral and index libraries as well as Data Analysis (Agilent Technologies). Unidentified compounds were manually annotated by their mass to charge ratio (m/z) and retention time (RT). Metabolites and compounds occurring in 50% of water controls as well as in 50% of chemical blanks were excluded from the metabolite list. Classification of metabolites was done according to their affiliation to natural substance classes.

3.1.3 – Plant traits and soil parameters

After excavation, plants were weighed both fresh and after drying. Root N and C content were obtained from root powder using a C/N-analyzer (vario EL cube; Elementar, Hanau, Germany). This information was provided by Geobotany group of the Institute of Biology / Geobotany and Botanical Garden from the Martin Luther University Halle-Wittenberg.

Soil variables of each plot were provided by a central soil sampling campaign in the Biodiversity Exploratories in 2014. Variables selected for each plot were soil pH, carbon and nitrogen content, which were determined as described by Solly *et al.*, 2014. Land-use intensity (LUI) was used as described by the index of Blüthgen *et al.*, 2012, using the values for 2014.

3.1.4 – RNA extraction

Rhizosphere and control samples were extracted in order to obtain bacterial RNA. Rhizosphere samples were made up of the soil particles and filters (cut up in small pieces).

3.1.4.1 – RNA / DNA co-extraction from rhizosphere samples

DNA / RNA extraction was performed as described by Lueders *et al.*, 2003 with modifications by Wüst *et al.*, 2016. Briefly, the samples were thawed on ice and transferred into a 2 ml screw cap tube with 0.7 g of sterilized zirconium/silica beads (diameter, 0.1 mm), 750 µl sodium phosphate solution and 250 µl TNS-Buffer (Tris-HCl, sodium chloride, SDS), and cells disrupted by beat-beating (2 times 6.5 $m \cdot s^{-1}$ for 45 s). After centrifugation, samples were extracted with phenol-chloroform-

isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1) and nucleic acids pelleted by the addition of polyethylene glycol and centrifugation. Pellets were washed with cold 70% ethanol and resuspended in 20 – 50 µl Tris-HCl buffer (pH 8.5). The quantity and quality of the coextracts were checked with NanoDrop ND-100 (Peqlab Biotechnologie, Erlangen, Germany). The RNA / DNA extraction was done with assistance of Alicia Geppert, DSMZ Braunschweig.

3.1.4.2 – DNA digestion

RNA was prepared by digestion of co-extracted DNA with RNase free DNase I (ThermoScientific, Waltham, MA, U.S.) according to the manufacturer's instructions. It was then precipitated with sodium acetate / isopropanol, washed with 70% ethanol and ressuspended in RNase free water. Concentrations of RNA were determined using the Quant-iT RiboGreen RNA Assay Kit (Life Technologies, Darmstadt, Germany) and a microtiter plate reader (Tecan Infinite 200 PRO; Männedorf, Switzerland).

3.1.4.3 – Reverse transcription

RNA extracts were treated with RiboLock RNase inhibitor (final concentration 1 U μl^{-1}; Fermentas) prior to reverse transcription PCR. For synthesis of cDNA from extracted RNA the GoScript Reverse Transcription System was employed according to the protocol of the manufacturer (Promega, Madison, WI, USA) using random hexamers.

3.1.5 – Preparation of 16S rRNA V3 amplicon libraries and sequencing

3.1.5.1 – Indexing PCR (Bartram method)

The V3 region of the 16S rRNA gene was amplified using modified primer pairs 341F (5'-CCTACGGGWGGCWGCAG-3') and 518R (5'-CCGCGGCTGCTGGCAC-3') (Muyzer *et al.* 1993) which contain Illumina adapter sequences and binding sites for sequencing primers. Additionally, the reverse primer included an index region of 6 nucleotides (Bartram *et al.* 2011). All samples were amplified in triplicates. The reaction mix (final volume of 50 µl) contained 10 µl PCR buffer (5x; GC Phusion buffer), 1 µl dNTP mix (10 mM each), 0.2 µl each of forward and reverse primers (50 µM each), 1.5 µl dimethyl sulfoxide (DMSO; 100% v/v), 1 µl Phusion High-Fidelity DNA Polymerase (2 U μl^{-1}; Thermo Scientific, Waltham, USA). Amplification proceeded by an initial denaturation step at 94°C for 5 min, followed by 20 cycles of 94°C for 15 seconds, 59°C for 15 seconds, 72°C for 15 seconds, and final extension step at 72°C for 7 minutes. Amplifications were carried out in a Veriti 96-well thermal cycler (Applied Biosystems, Foster city, CA, USA).

3.1.5.2 – Metaphor agarose purification of amplicons

Amplicons were purified in 2% Metaphor (Lonza group, Basel, Switzerland) agarose gels to allow separation of products from primers and primer dimers. Subsequently, the PCR products were cleaned with NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany) and quantified using Qubit® dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA, US).

3.1.5.3 – Illumina HiSeq sequencing

Quality was checked with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, U.S.) and 50 samples were pooled in equal amounts. Sequencing was performed on a HiSeq 2500 (Illumina, San Diego, CA, USA) in a paired-end run, yielding a total of 4.70×10^8 sequence reads of 100bp.

3.1.6 – Downstream processing of amplicons sequencing data

3.1.6.1 – Pre-processing of sequencing reads

Reads were assigned to the samples using the index of 6 bp, and downstream processing including the trimming to 100 bp, removal of primer dimers and adapters was done based on detection methods implemented in FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The remaining reads were joined using fastq-join (Aronesty 2013) and subsequently checked for chimeric sequences with UCHIME (Usearch 5.2.32 10) applying the GOLD database from ChimeraSlayer (http://drive5.com/otupipe/gold.tz) as reference.

3.1.6.2 – OTU clustering and taxonomy assignment

Data were analysed using QIIME 1.9.1 (Caporaso *et al.* 2010) and sequences were grouped into operational taxonomic units (OTUs) using 99% similarity identity with closed-reference approach. *De novo* clustering was not computationally feasible for this amount of sequences. Representative sequences, the most abundant sequences showing up in each OTU, were assigned taxonomy against the SILVA 128 database (Quast *et al.* 2013). The taxonomic profiles were normalized by the 16S rRNA gene copy number.

3.1.7 – Statistical analysis

All analysis was performed using the program R, version 3.2.3 (R Core Team 2017), with the exception of the determination of core OTUs, which was done with Qiime 1.9.7. The R package phyloseq (McMurdie & Holmes 2013) was used for determination of abundances at different

taxonomic hierarchies and α-diversity estimates with different measures (richness, Shannon and Simpson diversity indices). Rarefaction analysis was performed using the iNEXt package in R (Chao *et al.* 2014; Hsieh *et al.* 2016). Data for community structure analysis was normalized with Cumulative Sum Scaling (CSS) algorithm, and differences in bacterial community composition were based on weigthed UniFrac distances (phyloseq package). For root exudate dataset the β-diversity estimates were based on Bray-Curtis distances obtained with the R package vegan (Oksanen *et al.* 2017). Results for multivariable analysis were visualized by Non-Metric Multidimensional Scaling (*NMDS*). Variation in bacterial community composition or root exudate composition, explained by distinct variables (soil type, plant species, plant origin and soil compartment), was tested for significance using permutational analysis of variance (PERMANOVA, vegan package, 999 randomizations).

To determine significant differences in relative abundances or in alpha diversity metrics between groups, a multiple comparison analysis of variance was done (Tukey's all pair comparisons), with package multcomp (Hothorn *et al.* 2008). For comparisons between two groups, t test was employed. Taxonomic groups with strongest impact on differences between community structures were identified with SIMPER (Similarity Percentages), performed with the simper function from the vegan package. The determination of the differential relative abundances (fold changes) between variables was done with the DESeq2 package (Love *et al.* 2014), using Wald significance test.

In order to quantify the variation observed in the rhizosphere bacterial community structure within each soil type, explained by plant parameters, soil parameters and root exudates, a redundancy analysis (RDA) and partial RDA were performed using rda function from vegan package. Only soil types with 10 or more samples were selected. A forward selection approach was used to overcome highly collinear variables in the root exudates dataset, using ordistep function from vegan package. Results were confirmed by low values of variance inflation factors (VIF) (vif.cca function, vegan package).

Selection of rhizosphere enriched OTUs was done based on ANOVA analysis on the relative abundances between rhizosphere and bulk soil samples (only OTUs with a minimum of six data points for each compartment were used). Furthermore, these rhizosphere enriched OTUs were correlated with root exudates which occur at least 6 times per plant species, based on Pearson correlation. Both approaches were done using the stats package (R Core Team 2017).

All plots were generated using the R package ggplot2 (Wickham 2009), with the exception of ternary plots which were obtained using the ggtern package (Hamilton 2017) on relative abundance values, and of core OTUs plot which was obtained using the visualization tool Krona (Ondov *et al.* 2011).

3.2 – Mineral containers

The experimental set up, sampling, DNA extraction and qPCR measurements (3.2.1 – 3.2.4) were done by the Soil Biology group of the University of Hohenheim and the information was then provided for analysis in this study.

3.2.1 – Experimental set up

To investigate differences in bacterial colonization of minerals in soil, mineral-root container field experiments were installed the Biosphere Reserve Swabian Alb in Baden-Württemberg (Southern Germany), in the experimental plots of the German Biodiversity Exploratories project. Five low and five high land-use intensity (LUI) grassland sites were selected (Blüthgen *et al.* 2012). Sites were selected based on similar climatic conditions, soil type and pH. The soil type was classified according to the world reference base (WRB) as a rendzic Leptosol (Fischer *et al.* 2010).

The field experiment was installed on a 4 m × 4 m square in each grassland site. This area was divided into 64 equal subplots of 25 × 25 cm. In each site, three containers per sampling date were randomly distributed over the subplots. Each container was laterally inserted in the main root zone in a depth of 8 cm within an 18 × 18 cm square in the center of a subplot. Containers consisted of polyvinylchloride (PVC) with a diameter of 7 cm and a height of 1 cm. They were covered on top and bottom by a 50 µm mesh sized gauze (SEFAR NITEX®, Sefar AG Heiden, Switzerland), excluding root but enabling microbial ingrowth and colonization. Containers were filled with an artificial mineral mixture, selected according to Pronk *et al.*, 2012, and consisted of 71.4% illite (Inter-ILI Mérnöki Iroda, Hungary), 9.6% goethite (Bayferrox® 920, Lanxess Deutschland GmbH, Köln, Germany), 17% silt sized (Millsil W11 H) and 2% sand-sized (Quartz Sand Haltern, H33) quartz (Quarzwerke GmbH Frechen, Germany). It contained 1.49 µg NH_4^+ g^{-1} mineral DW, 0.04 µg NO_3^- g^{-1} mineral DW, 7.9 mg P kg^{-1} mineral DW and 436.6 mg K kg^{-1} mineral DW, 0.34 mg C_t g^{-1} DW, 0.12 mg N_t g^{-1} DW and had a pH of 5.2 (measured in 0.01 M CaCl2 solution). The volumetric proportion of the mineral mixture corresponded to the average soil texture of the studied grassland plots.

For the complex carbon addition experiment, containers were filled with 30.8 g mineral mixture and 0.27 g dried fine roots with a length of maximum 3 cm of *Dactylis glomerata* and *Lolium perenne* (IsoLife bv, Wageningen, Netherlands). Layers of mineral mixture and dried roots were alternately filled into the containers, with a layer of mineral mixture on top and bottom, to ensure equal distribution of material in all containers. The amount of root material and the origin plant species were selected according to the measured root density and the most common plant species at the sites (Regan *et al.* 2014).

For the simple carbon addition experiment, containers were filled with 30.8 g mineral mixture and loaded with artificial root exudates. Each container was loaded with 4.32 mg carbon, consisting of 68.35 % glucose and 31.65 % citric acid. These compounds were selected since they are commonly found as main root excreted substances. To avoid nutrient limitation, 10 % ammonium nitrate was also added. This value was selected taking into account the mean C/N ratio of the selected plots. To ensure attachment, a solution of 0.01 M $CaCl_2$ was additionally added. The mineral mixture was loaded by pipetting 15 ml of the artificial root exudate solution with uniform moistening.

3.2.2 – Sampling of mineral containers

For the complex carbon addition experiment, three randomly selected containers were excavated from each site after 29, 64, 210 and 373 days after the start of the experiment. Since the carbon sources added to the simple carbon addition experiment are easily metabolized, three randomly selected containers were excavated from each site in shorter intervals, after 14, 28, 60, 119 and 167 days after the start of the experiment. In addition to the mineral containers, soil samples were taken within a distance of 1 cm above and below the mineral containers (adjacent soil). Three control soil samples (control) were taken randomly from the free subplots, where no container had been added, in ~7 cm depth. Samples were transported to the in cooling boxes on ice bags and stored at 4 °C in the laboratory until further preparation in the laboratory.

Minerals from the containers were sieved (< 2 mm) and roots were separated by hand using tweezers. Adjacent and control soil samples were sieved (< 5 mm) and stones, roots and animals removed. The sample triplicates per site for minerals, roots, adjacent soil and control soil were homogenously mixed to yield one sample per site and sampling date for each sample type and aliquots stored at -24°C until further analysis.

3.2.3 – DNA extraction

Extraction of rDNA was done on 0.3 – 0.4 g soil (adjacent and control), minerals or roots using the FastDNA Spin Kit for soil (BIO101, MP Biomedicals, USA) and DNA quantified on a Nanodrop ND-2000 spectrophotometer (Thermo Scientific, USA).

3.2.4 – qPCR

Quantitative PCR (qPCR) was done on diluted extract with 5 ng μl^{-1} final DNA concentration. Dilution was done with ultrapure water. qPCR products on bacterial 16S rRNA DNA were amplified with a ABI prism 7500 Fast System (Applied Biosystems, USA) and SYBR Green was used as a detection system.

The reaction mixture contained 0.75 µl of primer 351f and 534r (López-Gutiérrez *et al.* 2004; Keil *et al.* 2011), 4.125 µl ultra-pure water, 0.375 µl T4, 7.5 µl SYBR Green, and 1 µl, respectively 1.5 µl, DNA template. Standard curves were set up using serial dilutions of known amounts of plasmid DNA which contained fragments of the analyzed genes. We calculated the absolute abundances as copies per gram soil. An initial denaturation step of 95°C for 10 min was followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s and extension at 72°C for 30 s.

3.2.5 – Preparation of 16S rRNA V3 amplicon libraries and sequencing

The V3 amplicon library preparation and sequencing of the six soil samples selected for cultivation was done as described for the rhizosphere samples (see 3.1.5 – 3.1.6), with a modification. Prior to the indexing PCR, the V3 region of the 16S rRNA gene was amplified using the primer pair 341F (5'-CCTACGGGWGGCWGCAG-3') and 518R (5'-CCGCGGCTGCTGGCAC-3') (Muyzer *et al.* 1993). Amplification was performed in a total volume of 50 µl with 11 µl 5x GC Phusion buffer, 1.1 µl dNTP mix (10 mM each), 1.7 µl DMSO, 0.2 µl of each primer (50 mM), 1 µl of template DNA and 0.6 µl Phusion Hot Start II DNA Polymerase (2 U μl^{-1}; Thermo Scientific, Waltham, USA). An initial denaturation step of 98°C for 30 s was followed by 15 cycles of denaturation at 98°C for 10 s, annealing at 58°C for 30 s and extension at 72°C for 30 s, and final extension step at 72°C for 10 minutes. The preparation of the 16S rRNA V3 amplicon libraries was done with assistance of Alicia Geppert, DSMZ Braunschweig.

3.2.6 – Statistical analysis

All analysis was performed using the program R, version 3.2.3 (R Core Team 2017). The R package phyloseq (McMurdie & Holmes 2013) was used for determination of abundances at different taxonomic hierarchies and α-diversity estimates with different measures (richness, Shannon and Simpson diversity indices). Rarefaction analysis was performed using the iNEXt package in R (Chao *et al.* 2014; Hsieh *et al.* 2016). Data for community structure analysis was normalized with Cumulative Sum Scaling (CSS) algorithm, and differences in bacterial community composition were based on weigthed UniFrac distances (phyloseq package). Results for multivariable analysis were visualized by Non-Metric Multidimensional Scaling (*NMDS*) and these were tested for significance using permutational analysis of variance (PERMANOVA, vegan package (Oksanen *et al.* 2017), 999 randomizations). To determine significant differences in relative abundances or in alpha diversity metrics between groups, a multiple comparison analysis of variance was done (Tukey's all pair

comparisons), with package multcomp (Hothorn *et al.* 2008). For comparisons between two groups, t test was employed.

In order to investigate temporal changes in relative abundance of mineral and root bacterial communities, OTUs with a relative abundance of more than 0.1% at any given timepoint were selected. For these, a temporal activity pattern was estimated based on the changes in relative abundance through time. Because some OTUs are much more abundant than others, the relative abundance values were adjusted to a 0-100 scale from the smallest to the largest value. Time was also adjusted to a 0-100 scale. Because for some OTUs at certain timepoints there are extreme outliers (behaviour in 1 – 2 plots is very different from the rest), which severely distort the overall pattern, these were removed. The temporal activity pattern was clustered automatically into 6 groups using the time-series clustering algorithm global alignment kernel (GAK) (Cuturi 2011) from the dtwclust package (Sarda-Espinosa 2018).
The influence of stochastic and deterministic assembly processes on the bacterial communities between timepoints was determined calculating abundance-weigthed β Mean Nearest Taxon Distances (βMNTD). Then a null modelling approach was employed which generates an expected level of βMNTD given a dominance of stochastic processes (random shuffling of species on the phylogenetic trees). For quantification of the magnitude and direction of deviation from the calculated βMNTD to the expected βMNTD, the β Nearest Taxon Index (βNTI) was calculated (Stegen *et al.* 2013). βNTI < −2 or > +2 indicates that the observed βMNTD deviates from the mean βMNTD obtained by null modelling by more than two standard deviations, and therefore it is considered to indicate significantly less than or greater than expected phylogenetic turnover, respectively, for a given pairwise comparison. A significant deviation (i.e., |βNTI|>2) indicates the dominance of deterministic processes and the lack of deviation (i.e., |βNTI| < 2) indicates the dominance of stochastic processes. The R code for this analysis is the one used by Stegen *et al.*, 2013 and is available on Github (https://github.com/stegen/Stegen_etal_ISME_2013).

All plots were generated using the R package ggplot2 (Wickham 2009).

3.3 – Cultivation

3.3.1 – Bulk soil sampling

Six soil samples were obtained from experimental plots of the Biodiversity Exploratories in May 2014 and were selected for cultivation since they reflected different soil characteristics and land use regimes (Table 2). These samples represented, for each of the Biodiversity Exploratories regions, one grassland and one forest soil. The sampling was done as part of a central soil sampling campaign in

the Biodiversity Exploratories in 2014. Each plot (experimental area in grassland/forest) was sampled in 14 distinct locations along two transects of 40 m for forests and 20 m for grasslands. The upper soil layer was collected from 0-10 cm depth using a split tube sampler (5 cm diameter). For the forest sites, the litter layer was removed before sampling. The soil corers were pooled for each plot and the composite sample was flash frozen in the field and kept in liquid nitrogen.

Table 2 – Selected properties of the soil samples selected for cultivation.

Grassland sample	Soil type	**pH**	**Use of the land**	**Animals**	**Plant cuts**	**Fertilization**
AEG08	Leptosol	6,53	Pasture	Sheep	0	No
HEG06	Stagnasol	5,74	Mowing meadow	Cattle	1	Yes
SEG01	Histosol	7,05	Meadow	No animals	3	Yes
Forest sample	**Soil type**	**pH**	**Type of forest**	**Main trees**	**Tree age**	**Management**
AEW08	Cambisol	6,12	Natural	Beech	Uneven aged	Unmanaged
HEW09	Luvisol	4,28	Commercial	Beech	Uneven aged stands	Selection cutting, moderately intensive
SEW02	Cambisol	3,6	Commercial	Pine	Young timber	Intensive

3.3.2 – RNA extraction, library preparation and sequencing

The RNA extraction, V3 amplicon library preparation and sequencing of the six soil samples selected for cultivation was done as described for the rhizosphere samples (see 3.1.4 – 3.1.6).

3.3.3 – Cell counting of soil samples

Total bacterial cell numbers were determined as described in Lunau *et al.*, 2005. Briefly, bacterial cells on 0.1 g of soil were fixed in 900 µl of 10 mM MES pH 5.5 with 1% glutaraldehyde (v/v). In order to break up particles and detach bacteria, 50 µl of the fixated cells were added to 1 ml MES (10 mM, pH 5.5) and 450 µl of 100% methanol. Subsequently, the sample was incubated in a 1.5 ml Eppendorf for 15 min at 35°C in an ultrasonic bath. Staining was performed by adding 0.5 ml of the suspension to 9.5 ml of MOPS (2 mM, pH 7) and 2 µl SYBR green (Life Technologies, Ltd, Paisley, UK; 1:5000 diluted), and incubation in the dark for 10 m with agitation. After incubation the suspension was filtered through a black 0.2 m polycarbonate filter. The filter was transferred to a microscope slide, and a cover slip with a drop of filtered mounting medium (1 ml of 3 x PBS, 9 ml of 100% glycerol and 25 mg DABCO) was placed upside down on it. Cells were counted with a Zeiss Axio Imager microscope at 1000 magnification. The filtration yielded 105 – 377 stained cells in the counting grid (125 x 125 µm), and 20 fields per sample were enumerated.

3.3.4 – High-throughput liquid medium dilution

Parallel liquid cultures were set up in microtiter plates, with 60 wells being inoculated per sample, per media and per dilution. Each well of the microtiter plates was filled with 180 µl medium and subsequently inoculated with 2 or 5 cells (20 µl, diluted in 10mM HEPES pH 7). Four different media were used, based either on soil solution equivalent (SSE) (Angle *et al.* 1991) or soil extract (BEX) (adapted DSM medium 12). Soil extract was obtained from a mixture of soils (collected in 2011) from the same regions (also separated for grassland and forest) of the soil samples used for cultivation. Six different soil extracts (BEX) were used and the soil samples were inoculated on the medium from the respective regions. The two basal media were amended with 3 mixtures of organic carbon substrates, producing 4 distinct media: SSE/HD1:10, BEX/HD1:10, SSE/Cmix and SSE/Polymermix. Per liter of medium, either (i) 0.5 g peptone, 0.1 g glucose, 0.25 g yeast extract (medium HD1:10), (ii) 1g cellulose, 1 g chitin, 1 g curdlan, 1 g pectin, 1 g xanthan, 1 g xylan (medium Polymermix) or (iii) 400 µl of 50 mM aminoacids (alanine, arginine, cysteine, glutamine, glutamate, glycine, histidine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, asparagin, aspartate, leucine, isoleucine and valine), 400 µl of 50 mM sugars (glucosamine, mannitol, N-acetyl-D-galactosamine, turanose, β-gentiobiose, glucose, xylose, arabinose, trehalose and rhamnose), 400 µl of 50 mM aromatic compounds (sodium benzoate and sodium salycilate), 400 µl of 50 mM tricarboxylic acid cycle intermediaries (lactate, succinate, citrate, malate, pyruvate, α-ketoglutarate and oxaloacetate), 400 µl of 50 mM fatty acids (formiate, acetate, propionate, butyrate and valeriate), and 1 ml 0.0005% Tween 80 (medium Cmix). All media were buffered with HEPES to pH 7, autoclaved and supplemented with 10 vitamin solution (1 ml L^{-1}; Balch *et al.*, 1979) and trace element solution (1 ml L^{-1}; Tschech and Pfennig, 1984).

After 6 weeks of incubation at room temperature, grown wells were identified by turbidity (except the ones growing on SSE/Polymermix, see 3.3.4.1) and the bacterial inventory in each well was determined by sequencing (see 3.3.4.2). Plates were kept at 8°C until plated. Aliquots of each enrichment were plated on the corresponding medium solidified with both purified agar (1.5% (w/v); OXOID™) or gelrite (0.8% (w/v)). After incubation for 4 – 6 weeks at room temperature, representative colonies were picked from each plate and purified.

3.3.4.1 – Detection of grown wells on SSE/Polymermix

The growth on SSE/Polymermix medium cannot be detected by change in turbidity since this medium is already turbid. For this reason, 4 µl of each inoculated well was mixed with 0.5 µl of a mixture of SYTO 9 and propidium iodide (LIVE/DEAD™ BacLight™ Bacterial Viability Kit, Invitrogen).

The dye mixture was prepared according to manufacturer's instructions and then diluted (1:40). Cells were visualized in a Zeiss Axio Imager microscope at 1000 magnification.

3.3.4.2 – Multiplexing of enrichments

After the incubation the bacterial community grew in each well were analysed by a barcoded Illumina paired-end sequencing method targeting the 16S ribosomal RNA V1-2 hypervariable region (Camarinha-Silva *et al.* 2014). First, 20 – 40 µl of the cultures were transferred to eppendorfs. After centrifugation, cells were collected and ressuspended in 20 µl of Tris-buffer (10 mM, pH 8). Three freeze/thaw cycles (5 min in 70% ethanol and ice bath/microwave followed by 5 min in Thermoblock at 99°C) were employed in order to disrupt the cells. The V1-2 region of the 16S rRNA gene was amplified using the primer pair 27F and 338R (Lane 1991; Etchebehere & Tiedje 2005). Amplification was performed in a total volume of 50 µl with 4 µl 5x HF Phusion buffer, 1 µl dNTP mix (10 mM each), 0.4 µl of each primer (10 mM), 1 µl of template DNA and 0.2 µl Phusion High-Fidelity DNA Polymerase (2 U/µl; Thermo Scientific, Waltham, USA). An initial denaturation step of 98°C for 30 s was followed by 20 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 15 s and extension at 72°C for 45 s and final extension step at 72°C for 7 minutes. One microlitre of this reaction mixture served as template in a second polymerase chain reaction (PCR) performed under the same conditions as described above, in order to add barcodes. The forward primer contains a 6-nt barcode (Meyer & Kircher 2010) and a 2-nt CA linker (Hamady *et al.* 2008). Again, 1 µl of this reaction mixture served as template in a third polymerase chain reaction designed to integrate the sequence of the specific Illumina multiplexing sequencing primers and index primers. This was performed as before, but only for 10 cycles. PCR products were cleaned with NucleoSpin® 96 PCR Clean-up kit (Macherey-Nagel, Düren, Germany) as per manufacturer instructions and quantified with the Quant-iT PicoGreen dsDNA reagent and kit (Invitrogen, Darmstadt, Germany). Sequencing was performed in an Illumina MiSeq (Illumina, San Diego, CA, USA). After sequencing, the reads were joined, and sequences were sorted into their respective samples based on their barcodes, where primers and barcodes were then trimmed from each read. Taxonomic assignment was done using the RDP classifier (Wang *et al.* 2007) and with this information the wells harbouring potential novel strains were selected for plating. The downstream processing of the sequences was done using a bioinformatic pipeline developed by Boyke Bunk, DSMZ Braunschweig.

3.3.5 – Biofilm targeting approach

The enrichment and isolation of biofilm-forming bacteria was done as described by Gich *et al.*, 2012. Strips consisting of polypropylyne, polystyrene, glass and steel, were inserted in 20 ml glass vials with 15 ml of SSE/HD1:10 medium (pH 7). Soil samples were diluted in HEPES buffer (10 mM, pH 7) and 10000 cells were inoculated per vial. Two replicates for each combination of sample and surface were established. Vials were incubated for 6 weeks at 20°C with shaking. Three sequential enrichments were done transferring one strip to a fresh vial where a sterile strip of the same material was inserted, positioned so the two strips do not contact (Figure 8). The biofilm formed over the surfaces was swabbed and spread onto SSE/HD1:10 solidified with gelrite (0.8% (w/v)). Cultures were purified by subsequent re-streaking.

Figure 8 – 20 ml glass vial used for the enrichment and isolation of biofilm forming bacteria. Two parallel steel strips are submerged in SSE/HD1:10.

3.3.6 – Direct plating

The direct plating approach was used for the enrichment of slow-growing oligotrophic bacteria which need interactions with other microorganisms for growth. For this, 100 mg of soil was diluted in 900 µl of 10 mM HEPES, pH 7.0, and further tenfold serial dilutions were performed. Subsequently, 100 µl of the 10^{-6}, 10^{-7} or 10^{-8} dilution was added to SSE/HP (pH 7) medium and spread with a Drigalsky spreader. This medium is based on SSE, supplemented with 0.1 g peptone, 0.1 g yeast extract, 1 ml L^{-1} 10-vitamin solution (Balch *et al.*, 1979) and 1 ml L^{-1} trace element solution SL-10 (Tschech and Pfennig, 1984), and was solidified with 0.8 % (w/v) gellan gum. Cultivation proceeded for 4 months at room temperature in darkness. After this period, single colonies were picked and subsequently re-streaked to obtain pure cultures. For maintenance of cultures SSE/HD1:10 (pH 7.0) was used.

3.3.7 – Identification of pure cultures

In order to identify the isolated cultures, the almost full-length 16S gene of strains was amplified directly by colony-PCR using primer pair 8f (5´-AGAGTTTGATCCTGGCTCAG-3´) (Turner *et al.* 1999) and 1492r (5´-GGTTACCTTGTTACGACTT-3´) (Lane 1991). PCR mixtures included 2.0 µL PCR buffer (10x), 0.8 µL MgCl2 (25 mM), BSA 0.4 µL (20 mg mL^{-1}), 0.4 µL dNTPs (10 mM each), 0.08 µL each forward and reverse primers (50 pmol μL^{-1}), 0.08 µL Dream Taq DNA polymerase (5 U μL^{-1} Thermo Scientific) and 1.0 µL template (a stab of each colony added to 20 µL of water) in a total volume of 20 µL. The thermal cycling program consisted of: (i) 10 min at 94 ºC; (ii) 32 cycles of 30 s at 94 °C, 30 s at 56 °C and 1 min at 72 ºC, and (iii) a final elongation step of 7 min at 72 ºC. PCR products were purified and sequenced using the above primer pairs and the internal primers 1055f (5´-ATGGCTGTCGTCAGCT-3´) (Lane 1991) and 341r (5´-CTGCTGCCTCCCGTAGG-3´) (Muyzer *et al.* 1993), and by Sanger sequencing employing the AB 3730 DNA DNA analyzer (Applied Biosystems, Foster City, CA) and the AmpliTaq® FS BigDye® Terminator Cycle Sequencing Kit. Subsequently, the 16S rRNA sequences were analysed with the online database EzBioCloud (Yoon *et al.* 2017).

3.3.8 – Characterization of *Luteitalea pratensis* HEG_-6_39^T

The new representative of *Acidobacteria* subdivision 6, HEG_-6_39^T, successfully isolated from HEG06 sample with the direct plating approach was characterized by a polyphasic approach and validly described as *Luteitalea pratensis* gen. nov, sp. nov.

3.3.8.1 – Chemotaxonomic characterization

Cells were observed under a light microscope (Zeiss Axio Lab. A1; Carl Zeiss, with AxioCam Mrm camera) after Gram (Gerhardt 1994), India ink and malachite green staining (Bast 2014). Transmission electron microscopy was done by the Ultrastructural Research Group from the Department of Biology of the Ludwig Maximilian University of Munich. Cells were frozen at high pressure and cryosubstituted to minimize artefact formation due to shrinking of cells during chemical fixation (Wanner *et al.* 2008).

Catalase activity was determined as described by Cowan *et al.*, 1993. Cytochrome c-oxidase activity was analysed using Bactident Oxidase (Merck).

The relationship to oxygen was assessed in triplicate in liquid anoxic SSE/HD1:10 at 28°C. Growth ranges and optima of temperature and pH were determined in triplicate in oxic, liquid SSE/HD1:10 medium (Foesel *et al.* 2013; Huber *et al.* 2014, 2016). Growth was tested between pH 1 and 12, and depending on the pH value MES, HEPES, HEPPS or CHES (10 mM each) (Sigma-Aldrich or Applichem)

were used as buffers. The temperature range for growth was tested between 0 and 45°C. Salt tolerance was determined in modified DSMZ medium 1124 (10 mM HEPES buffer, 0.1 ml 10-vitamin solution l^{-1} (Balch *et al.* 1979) and 1 ml trace element solution SL-10 l^{-1} (Tschech & Pfennig 1984), as the SSE/HD1:10 medium already contains different salts. NaCl concentrations between 0 and 10 % (w/v) were tested. The ability to metabolize different substrates was evaluated in three parallel tests using liquid SSE supplemented with 0.1 ml 10-vitamin solution l^{-1} (Balch *et al.* 1979) and 1 ml trace element solution SL-10 l^{-1} (Tschech & Pfennig 1984). Sugars, organic acids, keto acids, alcohols, amino acids, casamino acids, casein hydrolysate, laminarin, peptone, yeast extract and Tween 80 were added to the medium as sole carbon sources. The final concentrations of each substrate have been previously described (Huber *et al.* 2014, 2016). Solidified SSE medium supplemented with 0.005 % (w/v) yeast extract was used to investigate the degradation of complex substrates such as cellulose, chitin, starch, xylan, pectin and lignin (0.5 ml l^{-1} final concentration of each polymer). Suitable aqueous staining solutions were employed to determine the ability to degrade the polymeric substrates (Pascual *et al.* 2015). Exoenzyme activities, nitrate reduction, indol production, fermentation of glucose, b-galactosidase, arginine dihydrolase and urease activities, and gelatin and aesculin hydrolysis were determined using the API ZYM and API 20NE test systems (bioMérieux) following the manufacturer's instructions. The utilization of carbon substrates could not be determined using API 20NE as the medium employed is not suitable for growth of strain HEG_-6_39^T. Isoprenoid quinones were extracted from dried biomass with chloroform/methanol (2 : 1, v/v; (Collins & Jones 1981)) and subsequently analysed via HPLC (Tindall 1990). For fatty acid analysis, cells were grown for 10 days at 28°C in SSE/HD1:10 medium (pH 7.0). Fatty acids were extracted, saponified and methylated according to standard protocols of the Microbial Identification System (MIDI Inc.; version 6.1; (Sasser 2001)). Compounds were identified against the TSBA40 peak naming table database. The polar lipid composition was analysed by two-dimensional TLC (modified after Bligh and Dyer, 1959 and Tindall *et al.*, 2007). Susceptibility to antibiotics was assessed on SSE/HD1 : 10 agar plates using discs (Oxoid) containing the following antibiotics: penicillin G (10 units), oxacillin (5 µg), ampicillin (10 µg), ticarcillin (75 µg), mezlocillin (30 µg), cefalotin (30 µg), cefazolin (30 µg), cefotaxime (30 µg), aztreonam (30 µg), imipenem (10 µg), tetracyclin (30 µg), chloramphenicol (30 µg), gentamicin (10 µg), amikacin (30 µg), vancomycin (30 µg), erythromycin (15 µg), lincomycin (15 µg), ofloxacin (5 µg), norfloxacin (10 µg), colistin (10 µg), pipemidic acid (20 µg), nitrofurantoin (100 µg), bacitracin (10 units), polymyxin B (300 units), kanamycin (30 µg), neomycin (30 µg), doxycyclin (30 µg), ceftriaxone (30 µg), clindamycin (10 µg), fosfomycin (50 µg), moxifloxacin (5 µg), linezolid (30 µg), nystatin (100 units), quinupristin/dalfopristin (15 µg), teicoplanin (30 µg) and piperacillin/tazobactam (40 µg).

The almost full-length 16S rRNA gene was amplified directly by colony PCR using primer pair 8f (Turner *et al.* 1999) and 1492r (Lane 1991). Sequences of the purified PCR products were determined by Sanger sequencing employing the AB 3730 DNA analyser (Applied Biosystems) and the AmpliTaq FS Big Dye terminator cycle sequencing kit. Alignment of this 16S rRNA gene sequence (1506 bp) with those of the described species of *Acidobacteria* subdivisions 1, 3 and 6 was done with the SILVA Incremental Aligner (SINA) (Pruesse *et al.* 2012). Phylogenetic trees were calculated using the neighbour-joining (Kimura's two-parameter evolutionary model) and maximum-likelihood (GTR+I+G evolutionary model) algorithms using the MEGA 6.0 software (Tamura *et al.* 2013). The G+C content was 64.7 inferred from the full genome sequence deposited at GenBank under accession number CP015436 (Huang *et al.* 2016). Genomic representation of HEG_-6_39T genome was done with CGView Server (Grant & Stothard 2008). Pairwise 16S rRNA gene sequence similarity calculated using p-distance (MEGA 6.0 software (Tamura *et al.* 2013)).

In order to assess the niche occupation of HEG_-6_39^T, the near full length 16S rRNA was aligned by blast against the 16S rRNA gene amplicon-based microbial profiles of the Integrated Microbial Next Generation Sequencing (imngs) depository, at 99% similarity (Lagkouvardos *et al.* 2016).

3.3.8.2 – Genome sequencing, assembly and annotation

Genome sequencing was carried out on the PacBio *RSII* (Pacific Biosciences, Menlo Park, CA) using P6 chemistry. Genome assembly was performed with the "RS_HGAP_Assembly.3" protocol included in the SMRT Portal version 2.3.0, utilizing 97,934 postfiltered reads with an average read length of 13,738 bp. One complete chromosomal contig was obtained and trimmed, circularized, and adjusted to *dnaA* (*locustag*_00001) as first gene. A final genome quality of QV60 was determined during resequencing using the RS_BridgeMapper.1 protocol in SMRT Portal. In addition, genome sequencing was carried out on a HiSeq 2500 (Illumina, San Francisco, CA) in a 100-bp paired-end single-indexed run, resulting in 3.2 million paired-end reads. Quality improvement was performed with the Burrows-Wheeler Aligner (BWA) (Li & Durbin 2010) mapping the Illumina reads onto the obtained chromosome. Protein coding regions, 16S ribosomal RNA and tRNA genes were scanned and annotated with Prokka (Seemann 2014). The genome was also uploaded to the RAST (Aziz *et al.* 2008) service for comparative analysis. Metabolic pathways were identified online at KEGG (Ogata 1999) with subsequent manual gapfilling. Genome assembly and annotation was done by Thomas Riedel, DSMZ Braunschweig, and the subsequent analysis was done by Sixing Huang, DSMZ Braunschweig.

3.3.9 – Statistical analysis

All analysis was performed using the program R, version 3.2.3 (R Core Team 2017), unless otherwise stated. The R package phyloseq (McMurdie & Holmes 2013) was used for determination of abundances at different taxonomic hierarchies and α-diversity estimates with different measures (richness, Shannon and Simpson diversity indices) for soil samples and inventory of the grown wells of the high throughput liquid medium dilution approach. For the high throughput liquid medium dilution approach, the OTUs with less than 1% in relative abundance were eliminated from analysis since these should represent bacteria that did not grow in the conditions provided. Rarefaction analysis and species sample coverage estimates were performed using the iNEXt package in R (Chao *et al.* 2014; Hsieh *et al.* 2016). Data for community structure analysis was normalized with Cumulative Sum Scaling (CSS) algorithm, and differences in bacterial community composition were based on weigthed UniFrac distances (phyloseq package). Results for multivariable analysis were visualized by Non-Metric Multidimensional Scaling (*NMDS*). To determine significant differences in relative abundances or in alpha diversity metrics between groups, a multiple comparison analysis of variance was done (Tukey's all pair comparisons), with package multcomp (Hothorn *et al.* 2008). For comparisons between two groups, t test was employed. Correlation of relative abundance were determined for all pairs of genera growing in the wells and the species isolated with the high throughput liquid medium dilution approach and compared to null models with the netassoc package (Blonder & Morueta-Holme 2017). All graphs were obtained with the package ggplot2 (Wickham 2009), with the exception of the co-occurrence networks which were obtained with the igraph package (Csardi & Nepusz 2006) and the heatmaps which were obtained with the ggtree package (Yu *et al.* 2017).

V3 amplicon phylogenetic tree was obtained from QIIME (using FastTree, Price *et al.* 2010) and visualized with phyloseq. 16S rRNA sequences were trimmed with DNA Sequence Assembler (v4, Heracle BioSoft, www.DnaBaser.com), and alignments and phylogenetic trees, calculated using the neighbour-joining (Kimura's two-parameter evolutionary model) algorithm, were obtained with the MEGA 6.0 software (Tamura *et al.* 2013). The novel isolates near full length 16S rRNA gene sequences were aligned with BLAST against the amplicon datasets of the selected samples for cultivation, the rhizosphere and the mineral colonization.

3.4 – References

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Chapter 4 – Plant exudate and bacterial interactions in temperate grassland plant rhizospheres

4.1 – Results

4.1.1 – High throughput sequence read statistics

Next-generation amplicon sequencing of the V3 region of the 16S rRNA gene was employed to determine the structure of the bacterial community per sample. After quality filtering, denoising and chimera removal, approximately 398,000,000 sequences were obtained. A subset of approximately 97,000,000 sequences could be assigned using QIIME closed reference approach against the SILVA SSU Ref 128 database (clustered at 99% sequence similarity) at 99% sequence identity and were used for subsequent analysis. Sequences assigned to chloroplast were removed. After normalization of the counts, the sequences could be clustered in 34309 operational taxonomic units (OTUs). Near saturation was observed for the rarefaction curves performed for the OTUs present in rhizosphere, which indicated that our sequence inventory covered most of the taxa present in these samples (Figure 9).

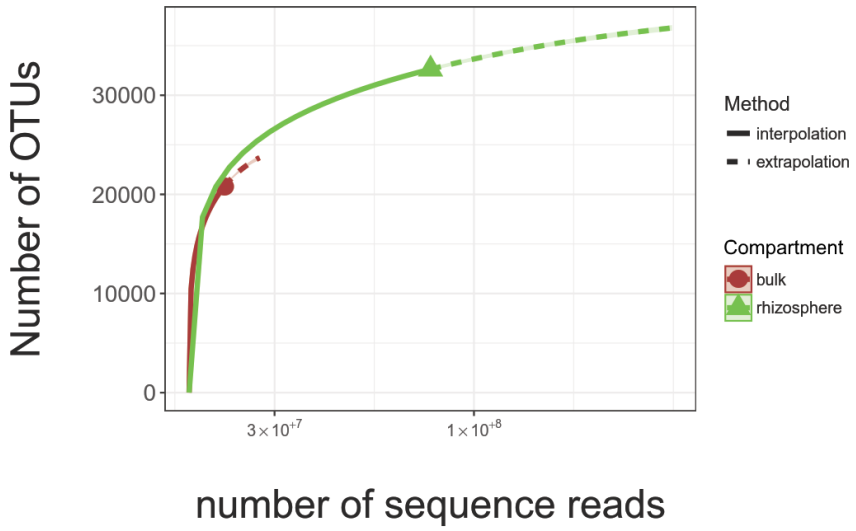

Figure 9 – Rarefaction curves for rhizosphere and bulk soil samples.

4.1.2 – Phytometer plant rhizospheres are similar to those of naturally occurring individuals

The bacterial communities in the rhizosphere of *Dactylis glomerata* and *Plantago lanceolata* were analysed in the plots where both phytometer plants and natural specimens occurred simultaneously. No apparent differences were observed for alpha diversity estimates between phytometer and the corresponding natural plants (Figure 10). In general, the rhizosphere of *Plantago lanceolata* was less species rich when compared to *Dactylis glomerata*.

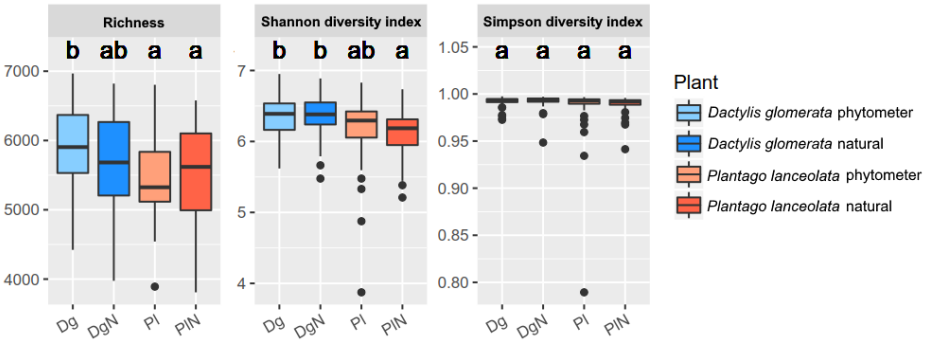

Figure 10 – Alpha diversity measures for bacterial communities at OTU level, for the two plant species where phytometer and natural individuals were collected. Letters denote significant differences ($p < 0.05$) as a result of multcomp test.

In a non-metric multidimensional scaling (NMDS) analysis, based on weighted UniFrac distances, there was no apparent separation of natural plants when compared to phytometers (Figure 11).

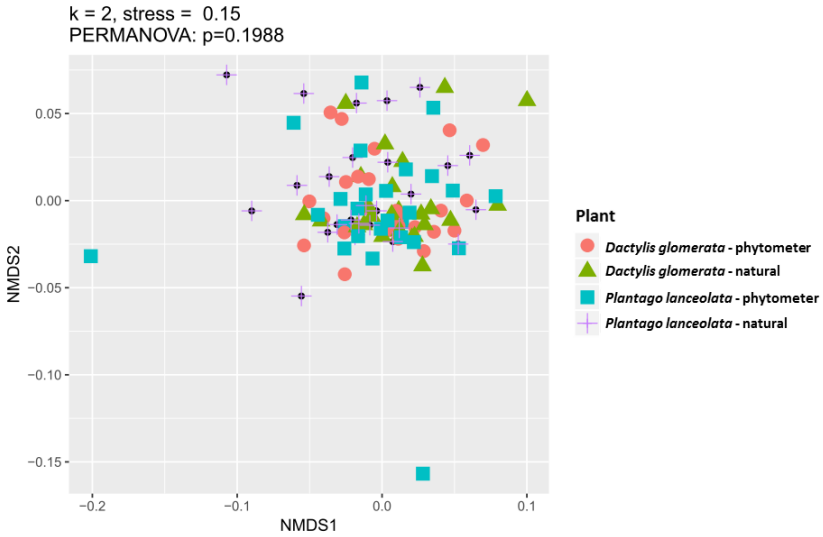

Figure 11 – NMDS plot comparing the bacterial composition of *Dactylis glomerata* and *Plantago lanceolata* rhizospheres based on weighted UniFrac distances at OTU level, between natural occurring specimens and phytometer plants.

In most cases the plants clustered by sampling plot (Figure 12) and not by either plant species or origin of the plants (natural versus phytometer). Together, this revealed that the bacterial communities in plant phytometer rhizospheres became identical to their natural counterparts. Therefore, only phytometer plants were taken into account for further analysis.

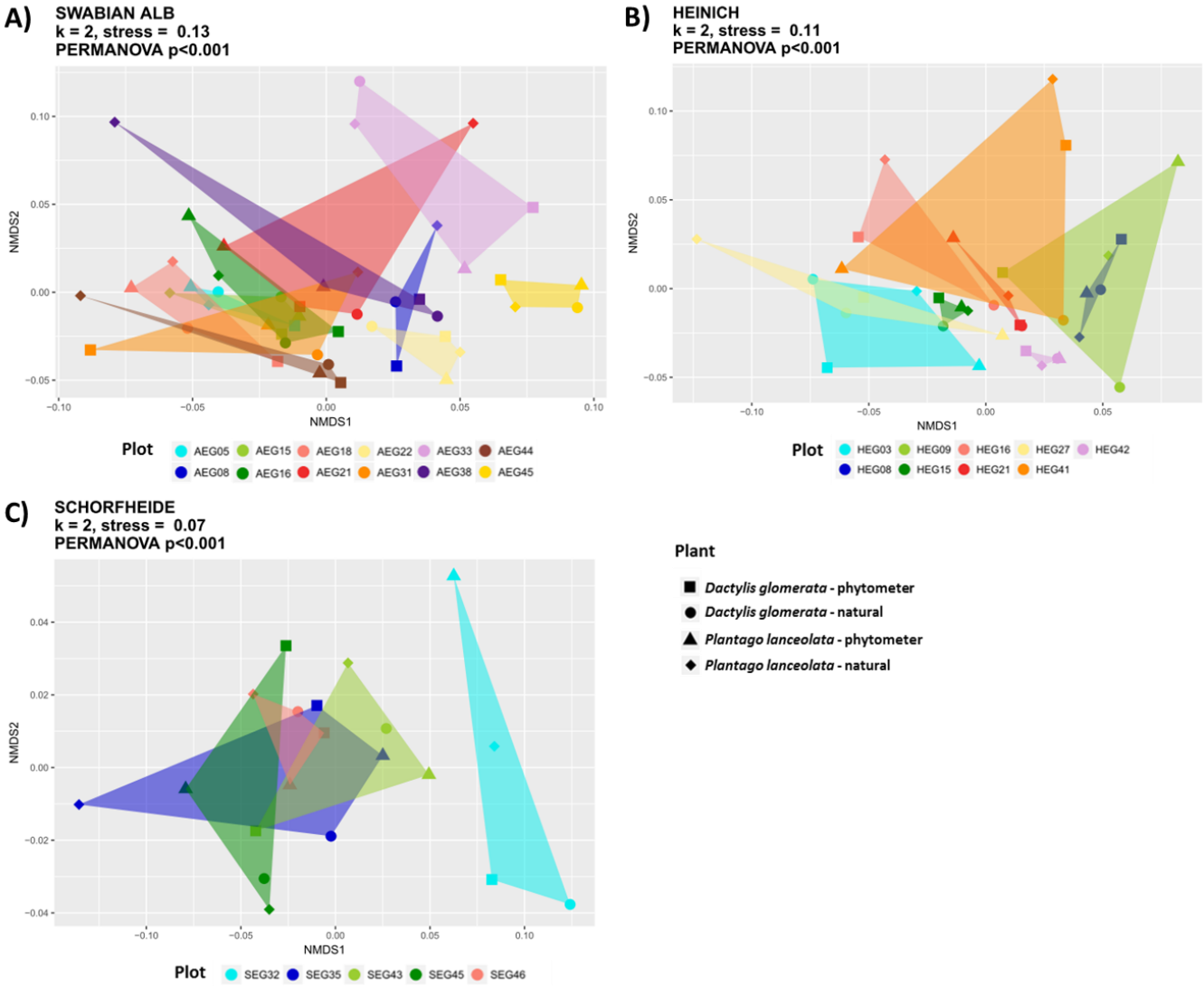

Figure 12 – NMDS plot comparing the bacterial composition of *Dactylis glomerata* and *Plantago lanceolata* rhizospheres based on weighted UniFrac distances at OTU level, between natural occurring specimens and phytometer plants in each of the Biodiversity Exploratories locations; A) Swabian Alb; B) Heinich; C) Schorfheide-Chorin. Samples from rhizospheres of *Plantago lanceolata* from plot 8 of Swabian Alb and plot 16 of Heinich were not included for better visualization, in the as they constitute extreme outliers.

4.1.3 – Geographic region drives bacterial community structure

Bacterial community richness and diversity did not differ between the three geographical locations investigated (Figure 13).

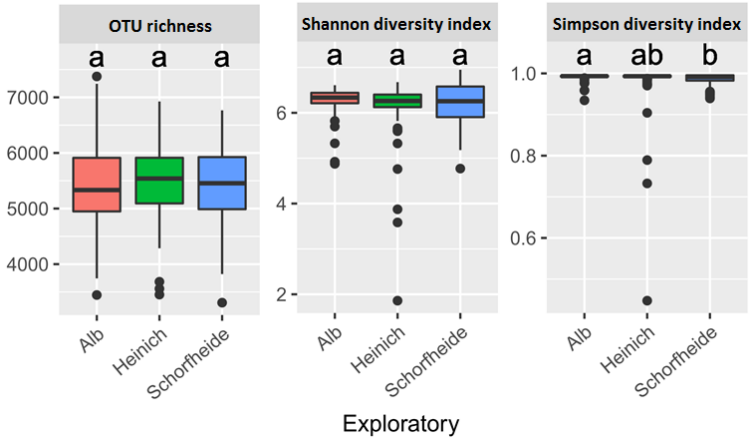

Figure 13 – Alpha diversity measures for bacterial communities at OTU level between the three Biodiversity Exploratories locations. Letters on top of each boxplot denote significant differences ($p < 0.05$) as a result of multcomp test.

The determinants of bacterial community composition were evaluated using NMDS based on weighted UniFrac distances at the OTU level. Bacterial community structures were significantly different at OTU level (PERMANOVA $p < 0.001$) between regions (Figure 14).

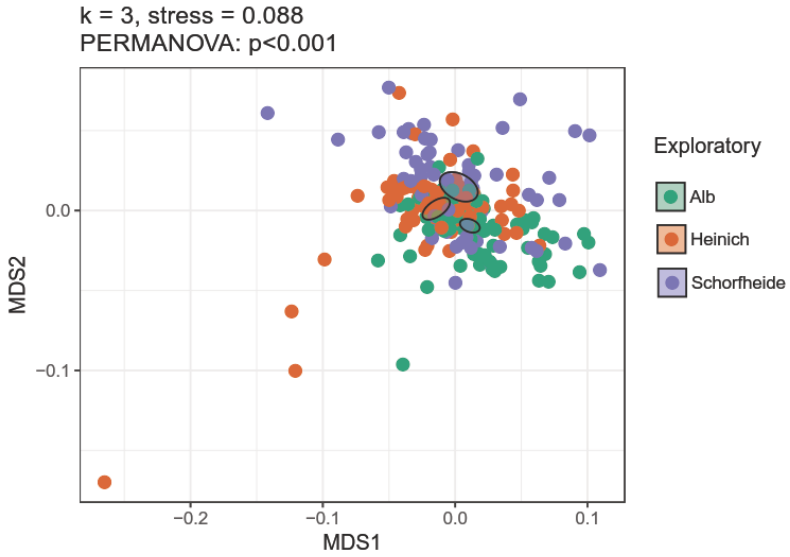

Figure 14 – NMDS plot of bacterial composition based on weighted UniFrac distances at OTU level, coloured by location. Ellipses denote 95% confidence intervals.

This is supported when looking at relative abundances, where we could identify OTUs which were shared exclusively between two or were unique to one location and many more which were preferentially associated with one of the locations. Nevertheless, the majority were shared between locations of the Biodiversity Exploratories (Figure 15A). At phylum (and proteobacterial class) level, the samples from Swabian Alb had a higher proportion of *Alphaproteobacteria* (24% average relative abundance versus 20.5% and 19.3%) and *Planctomycetes* (9.9% versus 7.79% and 8.5%), while the samples from Heinich harboured more *Actinobacteria* (42.6% versus 34.5% and 34.1%) and the samples from Schorfheide-Chorin were enriched with *Firmicutes* (12% versus 5.2% and 5.3%) (Figure 15B). In the case of Schorfheide-Chorin this enrichment with *Firmicutes* was likely due to the high abundance (4.2% of all Schorfheide OTUs) of a single OTU belonging to the *Bacillaceae* family.

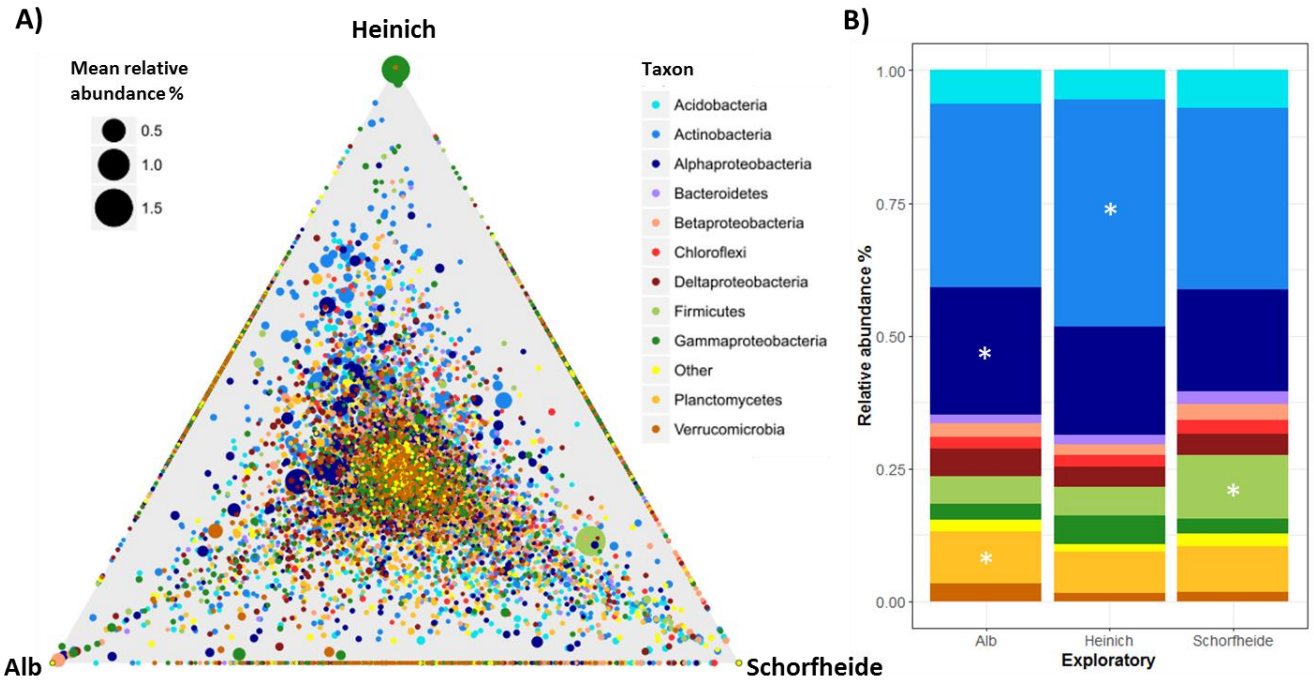

Figure 15 – Distribution of 34309 OTUs across regions. A) Ternary plot of OTU distribution across the three Biodiversity Exploratories regions. Each circle represents one OTU, and size, colour and position represent its mean relative abundance (across all samples), bacterial phylum and affiliation with the different regions, respectively. Points at the edges represent bacterial OTUs unique to the particular location. B) Average relative abundances of bacterial phyla and alphaproteobacterial classes across the regions. Taxa significantly enriched in one location when compared with the two others (detected with multcomp test ($p < 0.01$)) are marked with asterisks.

4.1.4 – Rhizosphere bacterial communities are distinct from bulk soil

Bacterial alpha diversity estimates were calculated for all rhizosphere and bulk soil samples and although there were significant differences regarding observed species richness (higher in rhizosphere when compared with bulk soil), no differences exist between rhizosphere or bulk soils across the location of Exploratories. Shannon diversity index revealed no differences between soil compartments (Figure 16).

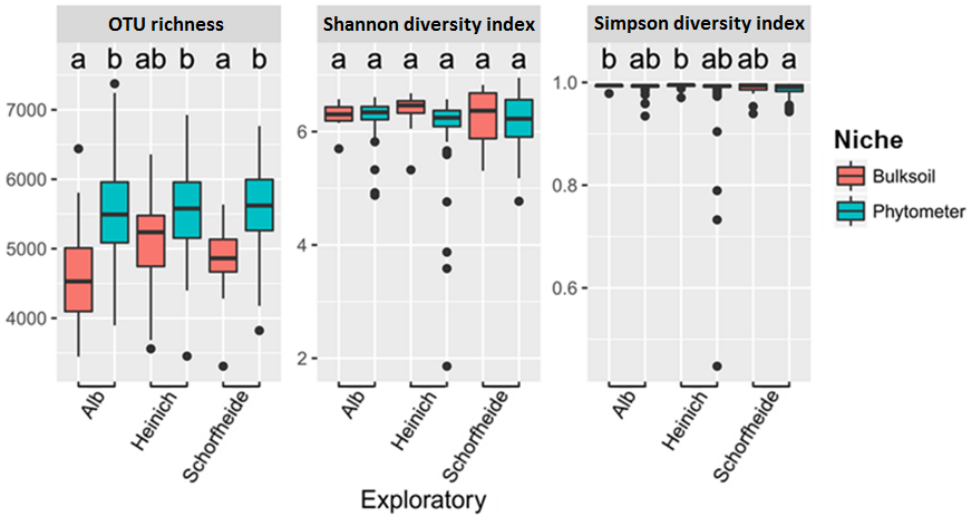

Figure 16 – Alpha diversity measures for bacterial communities at OTU level, within the different niches: rhizosphere (in blue) and bulk soil (in red). Letters on top of each boxplot denote significant differences ($p < 0.05$) as a result of multcomp test.

Based on analysis of community structure, rhizosphere communities were confirmed to harbour distinct communities when compared to the bulk soil (PERMANOVA $p < 0.001$) (Figure 17A).

Fifty bacterial phyla were identified, however only 8 were abundant (mean relative abundance > 1%), accounting for 98.2% of phytometer rhizosphere and 97.2% of the bulk soil. The rhizosphere bacterial communities (dominant phyla and proteobacterial classes) of all six phytometer plant species were significantly enriched for *Actinobacteria* (means of 39.1% versus 29.2%), *Alphaproteobacteria* (means of 22.2% versus 18.8%), *Bacteroidetes* (means of 2% versus 1.3%) and *Gammaproteobacteria* (means of 4.4% versus 1.5%), but depleted for *Acidobacteria* (means of 5 % versus 11.8 %), *Planctomycetes* (means of 7.9% versus 13%), *Chloroflexi* (means of 2% versus 3.5%), *Deltaproteobacteria* (means of 4% versus 6.3%) and *Verrucomicrobia* (means of 2.1% versus 3.4%). *Betaproteobacteria* (means of 2.5% versus 2.3%) and *Firmicutes* (7% versus 6.1%) are not significantly different between the two soil compartments (Figure 17B).

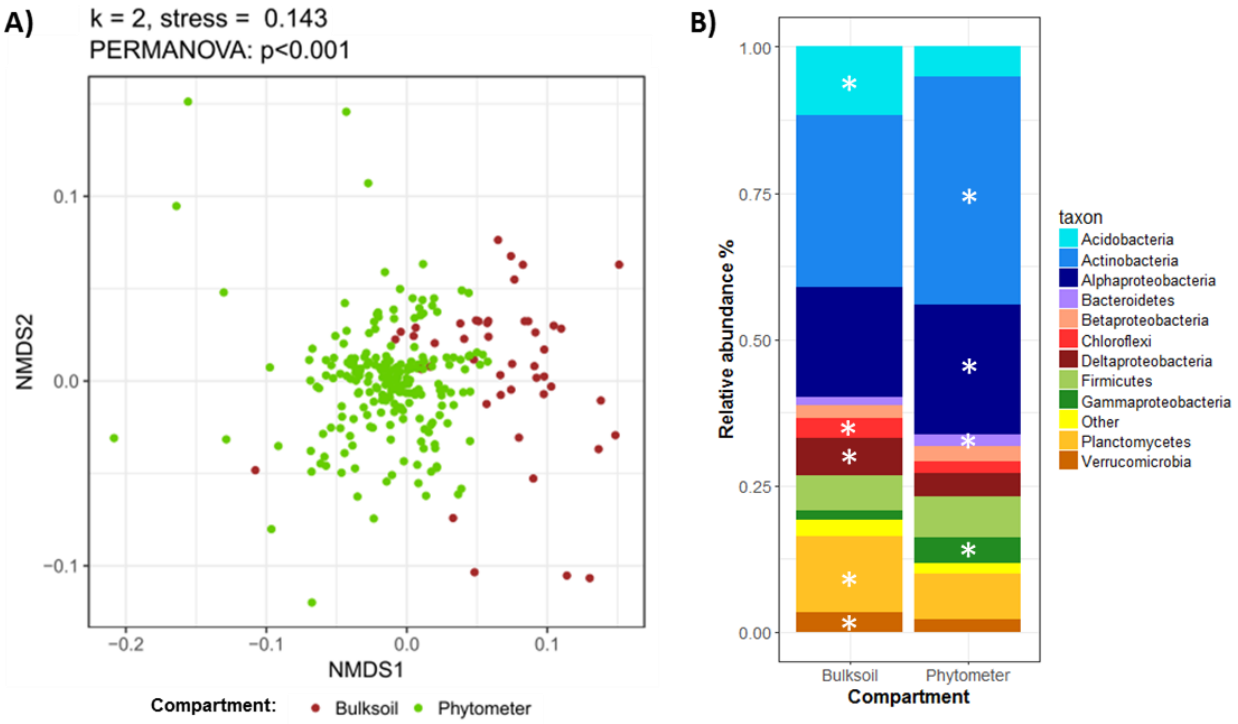

Figure 17 – Bacterial community structure and diversity between rhizosphere and bulk soil compartments. A) NMDS plot of bacterial composition based on weighted UniFrac distances at OTU level, coloured by compartment. B) Average relative abundances of bacterial phyla and proteobacterial classes in bulk soil or rhizosphere (of phytometer plants). Taxa with significant differential distribution (as result of t-test ($p < 0.01$)) are marked with asterisks.

Analysis of the quantitative increase or decrease in relative abundance in plant rhizospheres when compared to bulk soil, revealed that rare bacterial groups are the most affected. *Zixibacteria* (8 OTUs), GAL15 (5 OTUs) and *Omnithrophica* (12 OTUs) phyla were the ones affected most negatively, and were present 15.6, 10.5 and 5.1 fold less in rhizosphere samples, respectively. *Deinococcus-Thermus* (59 OTUs) and *Cyanobacteria* (385 OTUs) on the other end benefited from the rhizosphere compartment, and were present here 7.8 and 4 fold more frequently than in bulk soil, respectively (Figure 18). Only phyla and proteobacterial classes that were present in all samples and in a minimum of 10 samples for both rhizosphere and bulk soil compartments were taken into account.

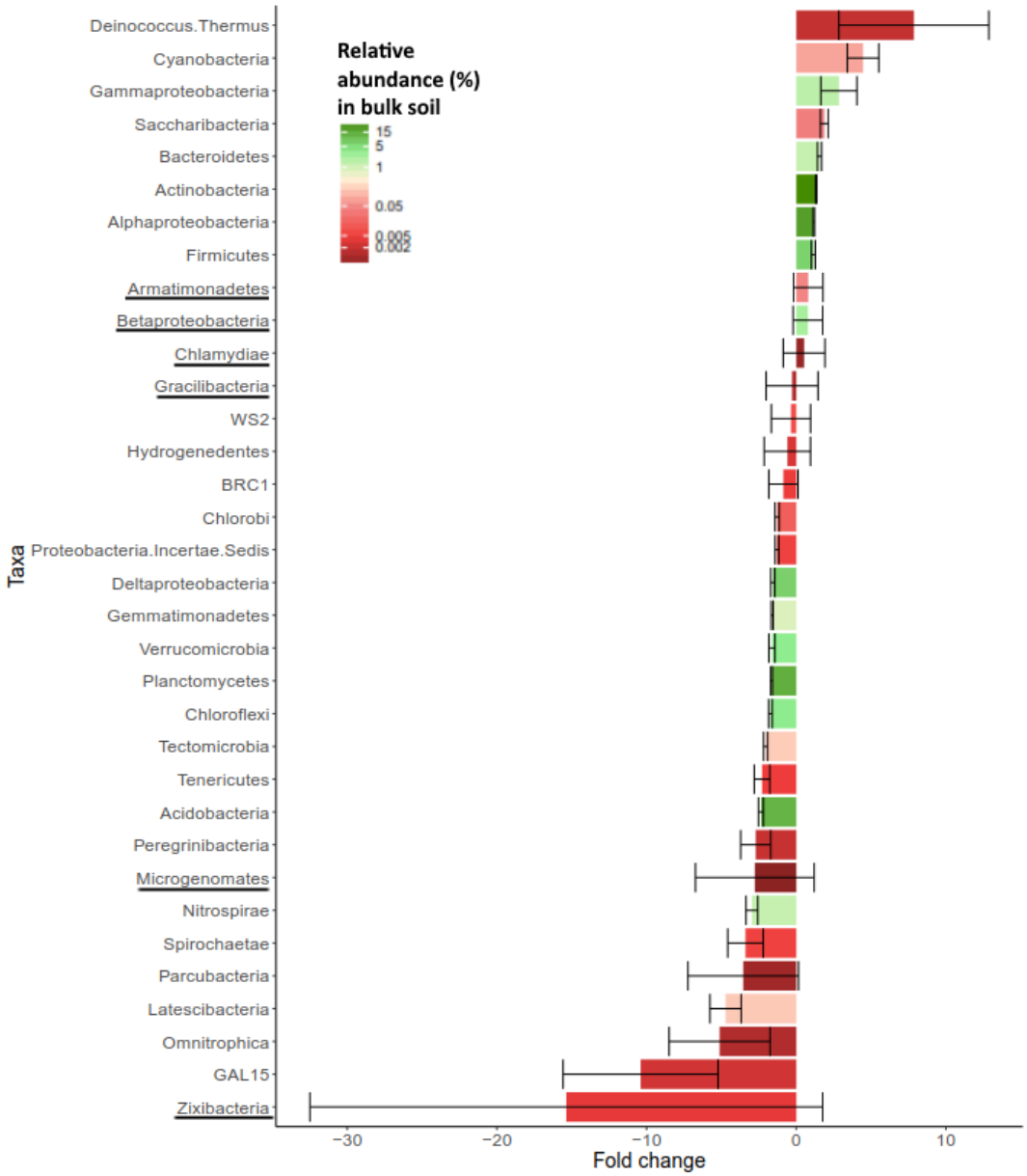

Figure 18 – Fold change differences (log2) between rhizosphere samples and bulk soil samples for the different phyla and proteobacterial classes. Bars indicate 95% confidence intervals as derived from t-tests (testing against a fold change of 0). Differences for underlined taxa are non-significant ($p > 0.05$) Colour represents relative abundance of the groups in bulk soil.

4.1.5 – Soil type is the major driver of rhizosphere community structure as opposed to plant species and root exudates

Since geographical distance can be a driver of bacterial communities, this effect was evaluated (Figure 19). Both across long regional distances (Figure 19A) or across local distances (Figure 19B and C) there was virtually no effect of distance in the similarity of bacterial communities (weighted UniFrac distances). An exception was registered for the Schorfheide location, where the more distant two samples, the more different were their bacterial communities (Figure 19D).

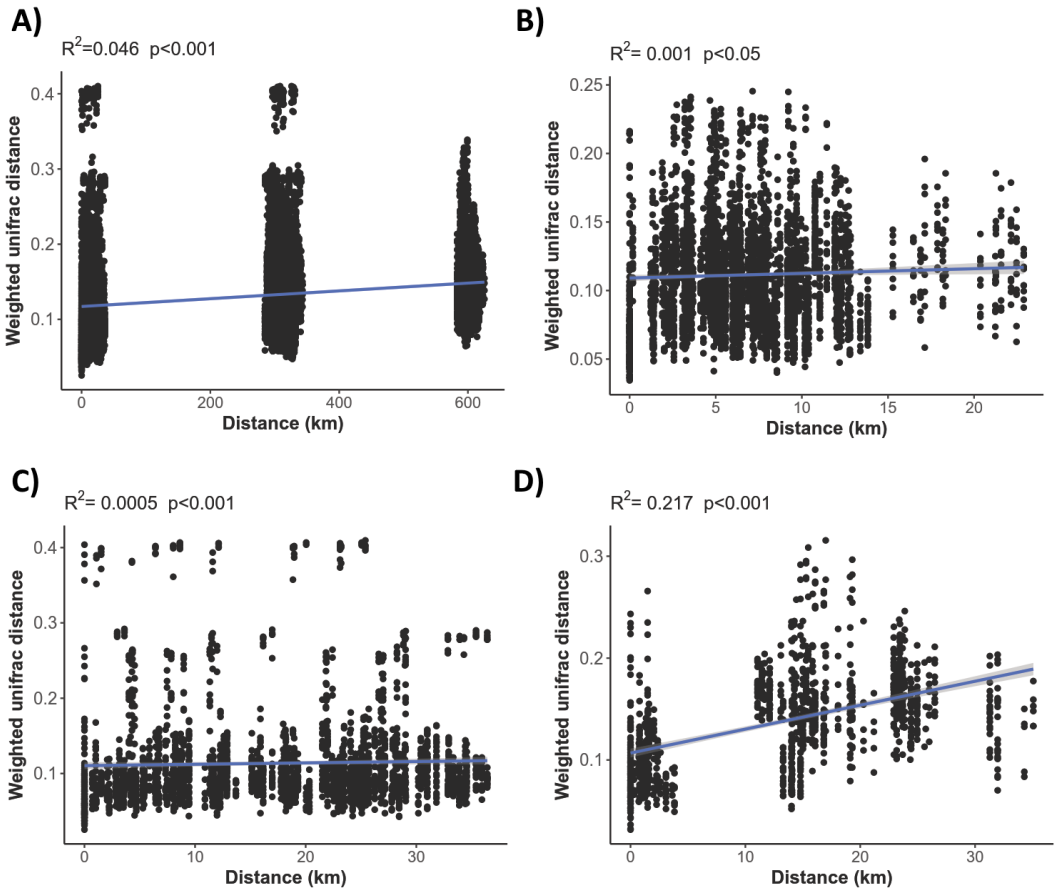

Figure 19 – Relationship between geographic distance (km) and similarity of rhizosphere bacterial communities (weighted UniFrac distance), for A) all samples and samples within each Biodiversity Exploratories location: B) Alb, C) Heinich and D) Schorfheide.

The effect observed for the Schorfheide region was further evaluated, accounting for soil type effects and it was observed that this relationship exists both within same soil types or across soil types (Figure 20). Nevertheless, this is a modest effect.

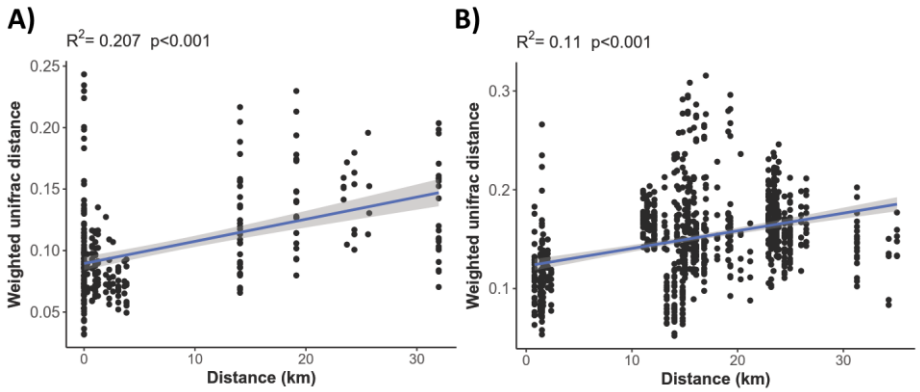

Figure 20 – Relationship between geographic distance (km) and similarity of rhizosphere bacterial communities (weighted UniFrac distance) for all rhizosphere samples of Schorfheide. This reflects all pairwise comparisons of samples A) within the same soil type or B) across different soil types.

Despite rhizosphere bacterial communities having similar richness (OTU level) values between soil types, distinct soils harboured distinct bacterial diversity, histosols being the most diverse and albeluvisols and luvisols showing lower diversity (Shannon diversity index, Figure 21A). Albeluvisol communities were also the more even of all soils. For plant species, differences could be seen in richness which was generally higher for grasses when compared to herbs. No differences were observed for diversity (Shannon diversity index) and eveness (Simpson diversity index) (Figure 21B).

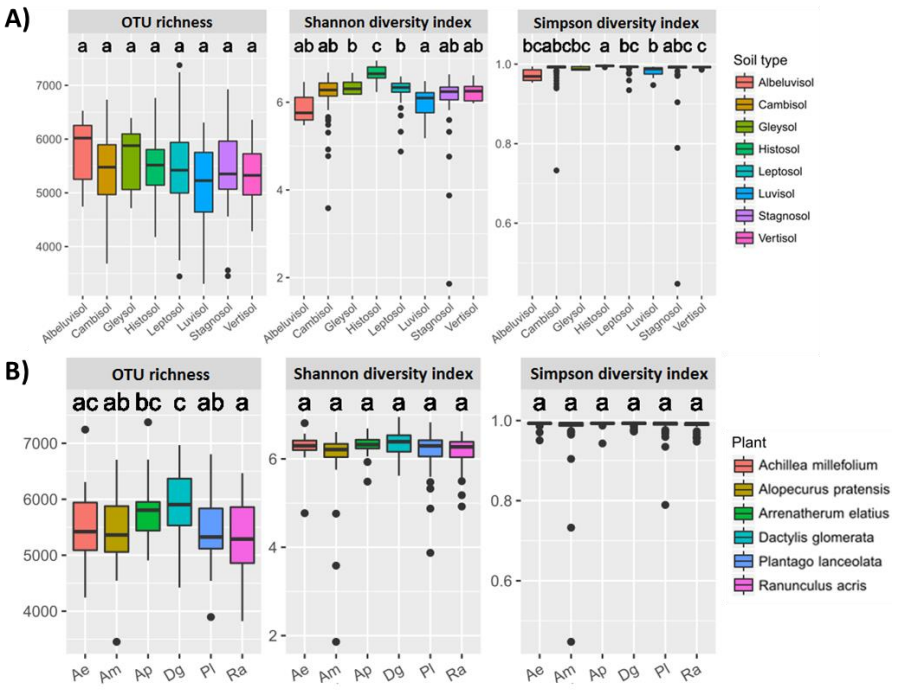

Figure 21 – Alpha diversity measures for rhizosphere bacterial communities at OTU level between A) soil types or B) plant species. Letters on top of each boxplot denote significance ($p < 0.05$) as a result of multcomp test.

In order to investigate the selective effect exerted by rhizosphere on the bacterial communities, the previous NMDS approach was employed again focusing on either soil type or plant species. Based on the 95% confidence intervals depicted in Figure 22A (filled circles) the rhizosphere communities were affected by different soil types (PERMANOVA $p < 0.001$). Plants growing in leptosols, histosols, stagnasols, vertisols and cambisols were especially distinct from luvisols, albeluvisols, and gleysols. In contrast, no conspicuous difference between the rhizosphere bacterial communities of the different plant species could be detected across all plots investigated (PERMANOVA $p = 0.2358$) (Figure 22B).

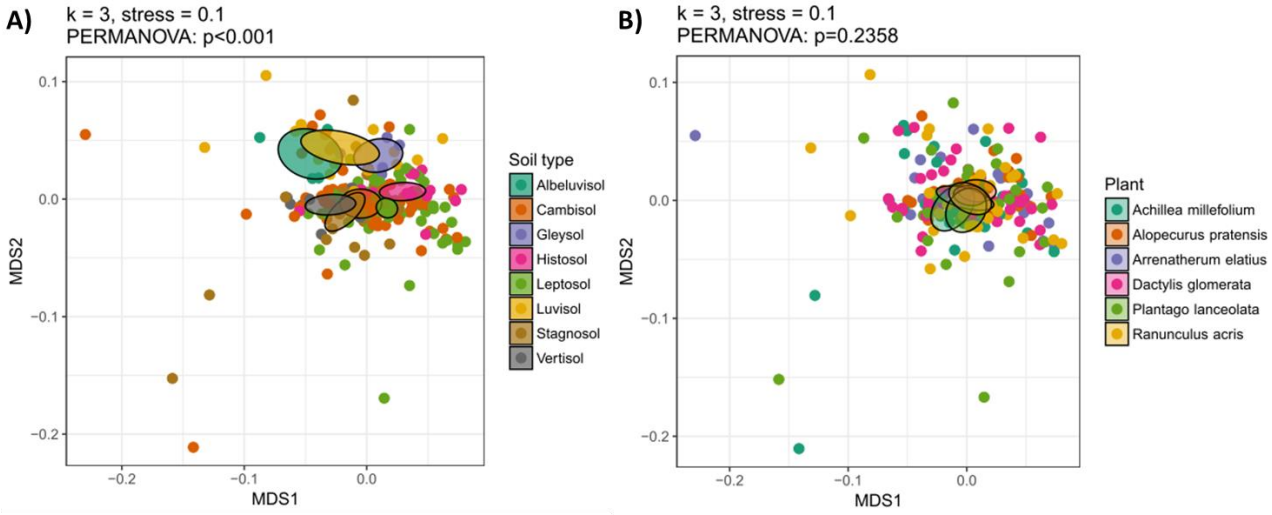

Figure 22 – NMDS plots of rhizosphere bacterial composition based on weighted UniFrac distances at OTU level, coloured by either A) soil type or B) plant species. Ellipses show 95% confidence intervals.

The same approach was employed for the analysis of 299 root exudate compounds obtained through Gas Chromatography Mass Spectrometry (GC-MS) measurements. Known structures were identified for 73 of these, which were mostly sugars, aminoacids and organic acids (Supplementary Table 1). As for bacterial communities, there was an influence of soil type in the composition of the root exudates (PERMANOVA $p < 0.05$), but in this case a plant influence was also registered (PERMANOVA $p < 0.001$) (Figure 23). The representation of the 95% confidence intervals in Figure 23B was not consistent with the results of the PERMANOVA analysis, due to the unavoidable limitation of ordination techniques which attempt to fit multidimensional data in a low dimensional space. Nevertheless, when adding the third NMDS axis (Figure 23C) the influence of plant species was clearer. *Plantago lanceolata* and *Ranunculus acris,* especially, seem to produce distinct sets of compounds compared to the other plant species (Figure 23B and C).

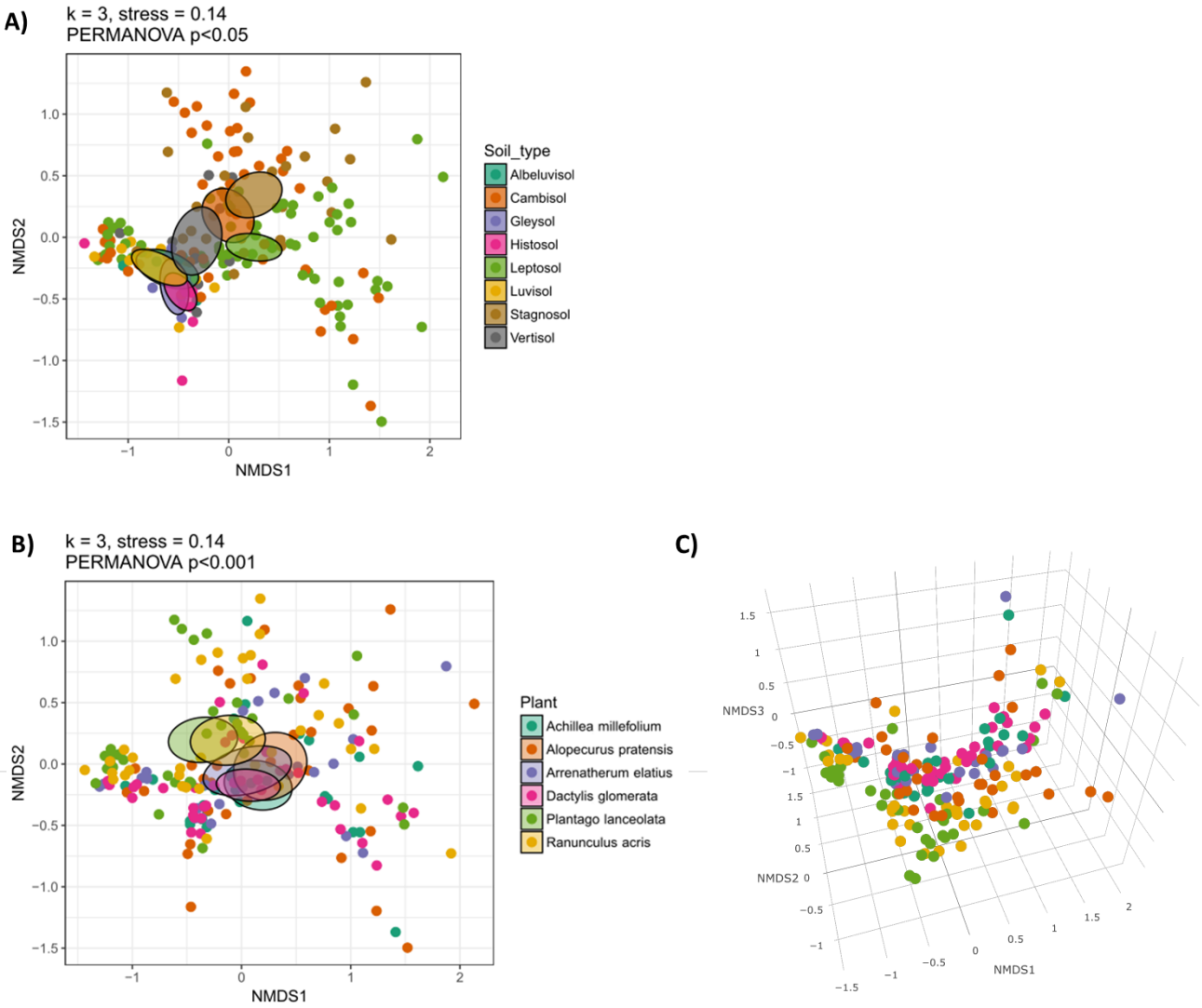

Figure 23 – Root exudate composition based on Bray-Curtis distances, coloured by either A) soil type or B + C) plant species. C represents the same NMDS plot as B but with the third NMDS axis also displayed. Ellipses show 95% confidence intervals.

Since the previous NMDS plots are unable to accurately represent the differences which exist between the different plant species (evaluated by PERMANOVA), and in order to confirm the previous results, we compared the mean weighted UniFrac distances within and across each pair of soil types and plant species (Figure 24). The mean values for each category (soil type or plant species) were used, because there are thousands of pairwise comparisons done which would produce significantly statistical differences just due to the large number of values used.

Differences in bacterial community composition increase substantially across different soil types whereas different plant species do not affect the microbial community composition (Figure 24, upper row). In the same fashion, we analysed the effect of soil type and plant species on the

composition of root exudates by comparison of Bray-Curtis distances across and between types. In contrast to bacterial communities, the chemical composition of root exudates is strongly affected by the plant species but not by the soil type (Figure 24, lower row).

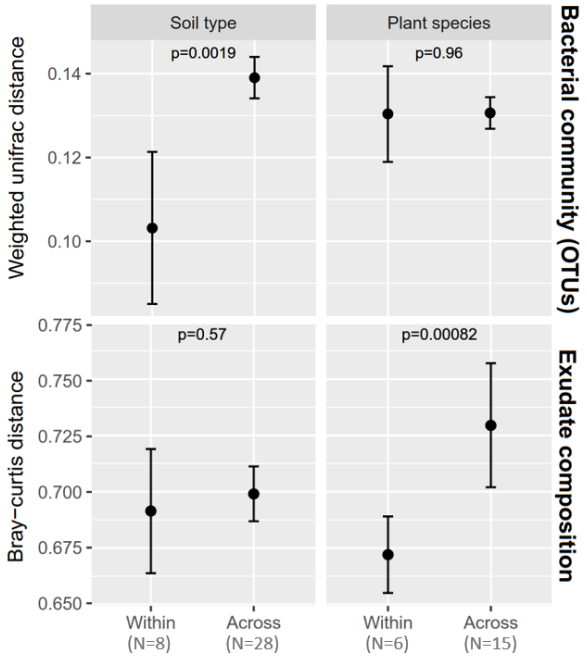

Figure 24 - The impact of either soil type or plant species categories on the similarity of bacterial communities or exudate compositions. “Within” indicates the mean of all pairwise comparisons of samples within given category (e.g., eight mean values for eight soil types) whereas “Across” indicates the mean of all pairwise comparisons of samples across any two categories (the comparison across all combinations of 8 soil types yields 28 mean values). Dots show the group-wise mean values, whiskers reflect 95% confidence intervals. The p-value results from t-tests.

To determine the quantitative contribution of physicochemical soil parameters (Supplementary Table 2), plant parameters (Supplementary Table 3) and root exudate composition (Supplementary Table 1) on the composition of rhizosphere bacterial communities, a variance partitioning analysis was employed. As the large number (N = 299) of (in part) highly collinear exudate compounds severely impedes computations, variance inflation values (VIFs) were determined and only compounds below the threshold of 10 were kept (Dormann *et al.* 2013), which resulted in a reduction of the number of exudates used to 61.

Soil properties explain most of the variance (27%), while the remaining variables hardly have an individual effect on the composition of rhizosphere bacterial communities (plant parameters 1%; root exudates 1%) (Figure 25). Moreover, the synergistic effects of root exudates and soil

parameters also explain a significant 8% of variance on the bacterial community structure. Nevertheless, 60% of variance still cannot be explained by the selected variables.

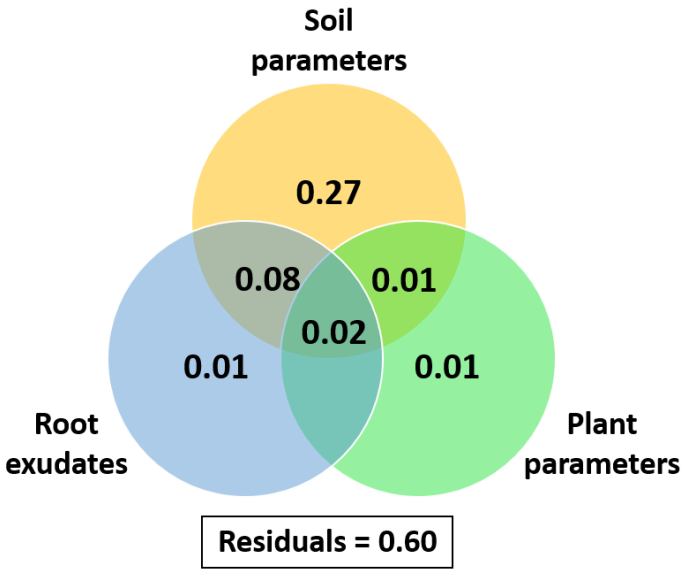

Figure 25 – Variance partitioning with proportion of explained variance of the bacterial OTUs.

Based on results, the support a direct impact of plant species on the structure of the bacterial rhizosphere community is very small. However, it is possible that these (possibly smaller) effects are masked by the strong effects of soil type. In order to exclude the strong effects of soil type, redundancy analysis (RDA) was employed on the bacterial rhizosphere composition within each soil type. Again, the effects were analysed separately for the physicochemical soil parameters (Supplementary Table 2), plant parameters (Supplementary Table 3) and root exudate composition (Supplementary Table 1).

Only soil types with 10 or more samples were chosen for this analysis (this reduced soil types from eight to six due to the removal of albeluvisol and gleysol). Since the exudate dataset is very large, and in order to better select those compounds which impact the rhizosphere bacterial community, a forward selection procedure was applied per soil. Again, variance inflation values (VIFs) of below 10 for the forward selected exudate data sets ensured that collinearity among exudate compounds was successfully removed. Forward selection resulted in different sets of exudates being chosen as best explaining the differences on bacterial community composition within the different soil types (Supplementary Table 4). Five exudates were retained for cambisol, leptosol and luvisol; 3 were retained for vertisol and 2 were selected in the case of histosol. Interestingly, forward selection

indicated that in stagnosol none of the exudate compounds were suited to explain variations in the bacterial rhizosphere community.

The results suggested that even within soil types the physicochemical soil parameter have a significant effect on the rhizosphere community composition. This was true for all soil types except luvisol (Table 3). However, the within-soil type analysis suggested also an influence of the root exudate composition on the bacterial rhizosphere composition, which was statistically significant in all soil types apart from cambisol. Congruent with the previous analysis, other plant parameters did not exert an effect on the rhizosphere community composition (Table 3).

As within a soil type both the soil parameters and the exudate composition affect the rhizosphere composition, we sought to quantify the separate effects of either soil parameters or exudate composition on the rhizosphere communities by controlling the variation of either one of them in partial RDA. However, the results from the partial RDA analysis (Table 4) do not differ from the results of the RDA (Table 4) which indicated that the analysed variables (exudates, soil and plant parameters) affect the bacterial communities independently from each other.

Table 3 –Redundancy Analysis (RDA) on the effects of soil parameters, plant parameters and exudates on phytometer rhizosphere bacterial community composition, within six different soil types. N represents the number of samples which represent each of the soil types. NA – Not possible to calculate. For stagnosol no compound was retained by forward selection (-). Significant effects are highlighted in bold.

		Leptosol (N=68)		Cambisol (N=48)		Stagnosol (N=30)		Histosol (N=16)		Luvisol (N=11)		Vertisol (N=10)	
Constraining dataset	**Response variable**	**Adjusted R^2**	**P value**	**Adjusted R^2**	**P value**	**Adjusted R^2**	**P value**	**Adjusted R^2**	**P value**	**Adjusted R^2**	**P value**	**Adjusted R^2**	**P value**
Soil parameters	OTUs	**0.1078594**	**0.001**	**0.1555849**	**0.004**	**0.256942**	**0.001**	**0.3619313**	**0.003**	0.246192	0.1	**0.4522128**	**0.013**
Plant parameters	OTUs	0.03278388	0.15	0.01807369	0.295	-0.1332623	0.712	0.3395943	0.063	0.6490868	0.12	NA	1
Exudates (F selection)	OTUs	**0.4009184**	**0.003**	0.2898489	0.075	-	-	**0.3158822**	**0.004**	**0.5559806**	**0.007**	**0.6558843**	**0.001**

Table 4 –Partial Redundancy Analysis (RDA) on the effects of soil parameters, plant parameters and exudates on phytometer rhizosphere bacterial community composition, excluding the combinatorial effects of the same variables, within six different soil types. N represents the number of samples which represent each of the soil types. NA – Not possible to calculate. For stagnosol no compound was retained by forward selection (-). Significant effects are highlighted in bold.

			Leptosol (N=68)		Cambisol (N=48)		Stagnosol (N=30)		Histosol (N=16)		Luvisol (N=11)		Vertisol (N=10)	
Constraining dataset	**Response variable**	**Effect removal**	**Adjusted R^2**	**P value**	**Adjusted R^2**	**P value**	**Adjusted R^2**	**P value**	**Adjusted R^2**	**P value**	**Adjusted R^2**	**P value**	**Adjusted R^2**	**P value**
Soil parameters	OTU table	Plant parameters	**0.1078594**	**0.001**	**0.1555849**	**0.005**	**0.256942**	**0.009**	**0.3619313**	**0.001**	0.246792	0.098	**0.4522128**	**0.036**
Soil parameters	OTU table	Exudates (F selection)	**0.1078594**	**0.001**	**0.1555849**	**0.001**	-	-	**0.3619313**	**0.001**	0.246192	0.097	**0.4522128**	**0.025**
Plant parameters	OTU table	Soil parameters	0.03278388	0.159	0.01807369	0.287	-0.1332623	0.717	0.339543	0.082	0.6490868	0.1	NA	1
Plant parameters	OTU table	Exudates (F selection)	0.03278388	0.154	0.01807369	0.29	-	-	0.3395943	0.078	0.6490868	0.134	NA	1
Exudates (F selection)	OTU table	Soil parameters	**0.4009184**	**0.002**	0.2898489	0.078	-	-	**0.3158822**	**0.006**	**0.5559806**	**0.005**	**0.6558843**	**0.001**
Exudates (F selection)	OTU table	Plant parameters	**0.4009184**	**0.01**	**0.2898489**	**0081**	-	-	**0.3158822**	**0.01**	**0.5559706**	**0.02**	**0.6558843**	**0.002**

4.1.6 – *Actinobacteria* and *Alphaproteobacteria* dominate rhizosphere core communities

Since we could not find a differential influence of plant species on the composition of rhizosphere bacterial communities, a core OTU analysis was performed for all plants in order to identify the bacteria consistently present in the rhizosphere of all plants. We could identify 473 OTUs that are present in 100% of the rhizosphere samples. These belong mainly to the phylum *Actinobacteria* phylum and the class *Alphaproteobacteria* and represent 58.3% of the total sequence reads of the phytometer rhizosphere bacterial community (Figure 26). Nevertheless, they constitute only a minority of all detected OTUs (0.016%).

Figure 26 – Krona plot with the 473 OTUs present in all plant rhizospheres. Circles from inside out reflect the different taxonomic levels: Phylum, Class, Order and Family. Percentages reflect the relative contribution of each group from all the core rhizosphere OTUs.

The ten most abundant of these OTUs belong to the genera *Bradyrhizobium*, *Bacillus, Variibacter, Streptomyces, Microlunatus, Nakamurella* and *Microbacterium*, and collectively make up to 15.1% of the total phytometer rhizosphere bacterial community (Table 5).

Table 5– Top 10 most abundant rhizosphere core OTUs.

Average relative abundance %	OTU	Genera
2.73	AM114522.1.1441	*Bradyrhizobium*
2.63	EF111142.1.1292	*Bacillus*
1.75	AM935458.1.1305	*Variibacter*
1.43	AJ316140.1.1445	*Streptomyces*
1.30	EU132893.1.1317	*Pseudonocardia*
1.23	JF167768.1.1343	*Streptomyces*
1.20	EU132629.1.1296	*Microlunatus*
1.11	EF018802.1.1366	*Nakamurella*
0.93	KF098235.1.1340	*Microlunatus*
0.83	DQ870743.1.1232	*Microbacterium*

4.1.7 – A small set of OTUs differ between plant species

Despite being very similar between plants, several differences between the different rhizosphere bacterial communities can be found at OTU level. 6885 OTUs were found to be exclusively present in a particular plant species (Table 6) but none of these OTUs was present in the majority of the individuals of the particular species. Moreover, these unique OTUs represent a minority of only ~2% of the total rhizosphere bacterial community for each plant species.

Table 6 – Number of unique OTUs in the different plant species.

Plant species	Nº unique OTUs
Arrenatherum elatius	*922*
Achillea millefolium	*1107*
Alopecurus pratensis	*991*
Dactylis glomerata	*1703*
Plantago lanceolata	*1121*
Ranunculus acris	*1039*

Since the OTUs occurring uniquely in a specific plant species rhizosphere are not present in the majority of the individuals of that species, this probably does not explain the differences observed between rhizosphere bacterial communities. Therefore, an enrichment or depletion in relative abundance of an OTU in a specific plant species rhizosphere, when compared to all other plant species rhizospheres was evaluated. A small subset of 27-71 OTUs that specifically responded, positively (> 1 fold log2) or negatively (< -1 fold log2), were found for each plant species (Figure 27).

The number of OTUs which responded positively (enriched) to a specific plant species ranged from 15 to 35 and reflected an average relative abundance of 0.12% to 0.57%. Most of these OTUs were rare, with only 20 having relative abundance values higher than 0.01% in the specific plant species rhizosphere. Differences could be observed between plants regarding taxonomical affiliation of positively associated bacteria. 15 OTUs belonging to *Hymenobacter* responded exclusively to *Achillea millefolium*; 5 OTUs of *Chloroflexi* and 3 OTUs of *Geobacter* were only detected in *Alopecurus pratensis*; 7 OTUs of *Mucilaginibacter* were associated with *Dactylis glomerata*; 2 OTUs of *Methylobacterium*, 2 OTUs of *Hyphomicrobium* and 2 OTUs of *Ideonella* were enriched in *Plantago lanceolata*; and 3 OTUs of *Planococcaceae* were only present in *Ranunculus acris*. Though not exclusive, *Arrenatherum elatius* harboured more OTUs (4) from *Enterobacteriaceae* than any of the other plants.

The number of OTUs which responded negatively (depleted) to a specific plant species ranged from 9 to 44 and reflected an average relative abundance of 0.025% to 0.44%. As for the OTUs which responded positively to a particular plant species, the majority of these OTUs were low abundant with only 23 of them having relative abundance values higher than 0.01% in the specific plant species rhizosphere. Although there were not so many distinct associations as in the positive responders, some could be observed. 6 OTUs of Pseudomonas were exclusively negatively associated with *Alopecurus pratensis* while 2 OTUs of *Friedmanella* were only detected to be depleted in *Alopecurus pratensis*. Although not exclusively, *Achillea millefolium* repeled more OTUs (3) belonging to *Acidobacteria* subdivision 6, while *Ranunculus acris* had negative effects on *Mucilaginibacter* (4 OTUs).

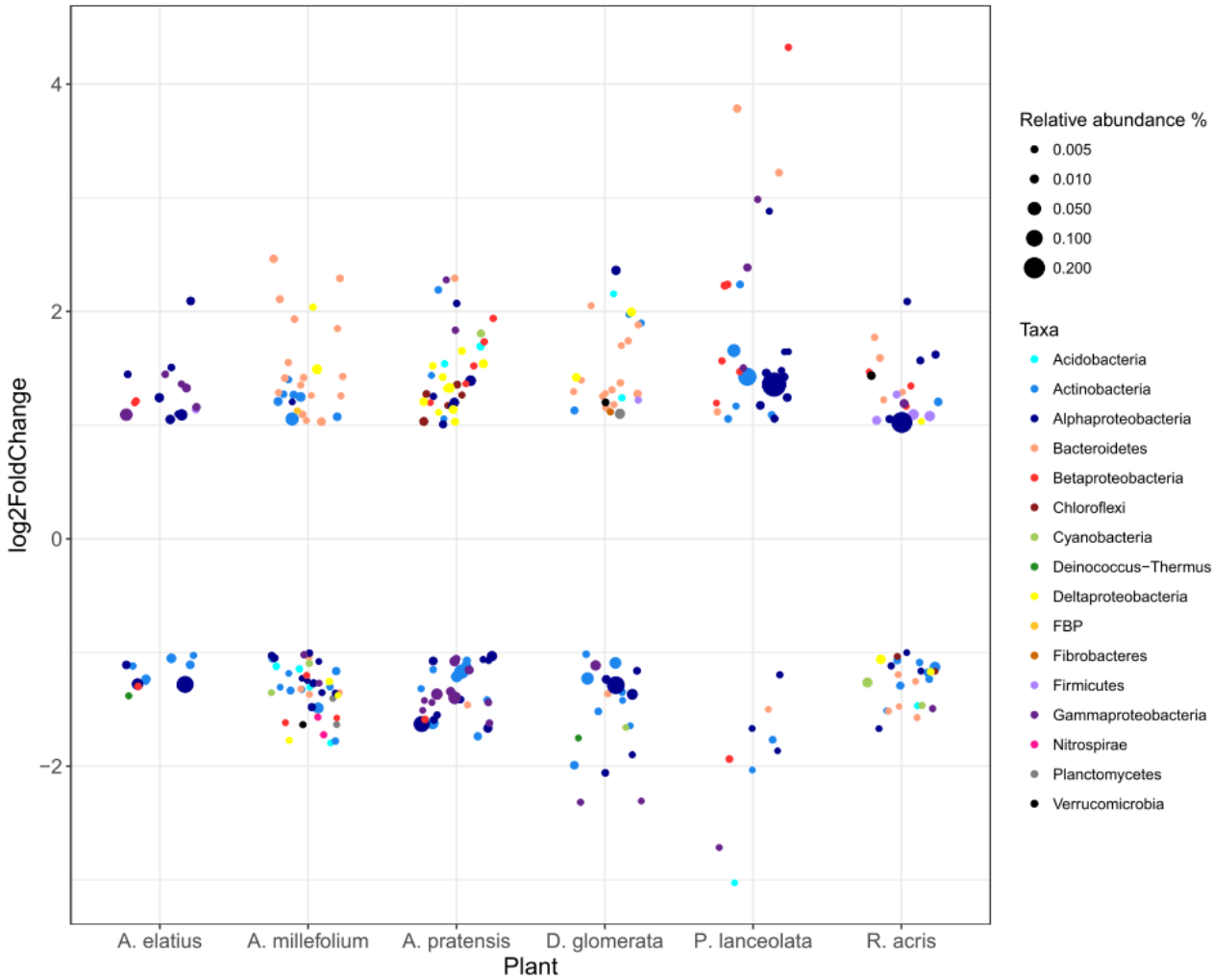

Figure 27 – Bacterial OTUs which responded significantly by relative abundance change according to one plant species. Each point represents an OTU with the size of the points being proportional to the mean relative abundance of OTUs in the specific plant rhizosphere. Colours indicate the taxonomic affiliation. Only significant values of fold change (log2) higher than 1 or lower than -1 ($p < 0.05$) are displayed.

4.1.7 – Individual root exudates from particular plants are associated with specific bacteria

To resolve the links of individual exudate compounds with rhizosphere bacterial communities, OTUs associated with rhizosphere were selected. An ANOVA analysis (with a selection of a minimum of six data points for each plant species and bulk soil) on the relative abundances of OTUs was performed between rhizosphere and bulk soil samples, and comprised 1001 OTUs that were identified as significantly enriched in the rhizosphere of at least one plant species. These OTUs were correlated with root exudates (based on Pearson correlation), employing a threshold of a minimum of 6 occurrences per plant species, which reduced the exudate dataset to 197 compounds.

This selection was made for all bacterial phyla (and proteobacterial classes) with an average relative abundance above 1%. *Acidobacteria* were excluded from subsequent analysis since they contributed less than 10 OTUs that were positively associated with exuded compounds. Positive correlations with 100 of the exudate compounds were obtained for 912 OTUs. *Actinobacteria* contributed 294 OTUs, *Alphaproteobacteria* 217 OTUs, *Bacteroidetes* 71 OTUs, *Betaproteobacteria* 38 OTUs, *Chloroflexi* 18 OTUs, *Deltaproteobacteria* 64 OTUs, *Firmicutes* 39 OTUs, *Gammaproteobacteria* 51 OTUs, *Planctomycetes* 99 OTUs, *Verrucomicrobia* 24 OTUs.

The specific relationships between OTU, plant species and exudate were evaluated and although many are similar, some taxa were found to be differentially attracted to specific compounds (Figure 27). *Gammaproteobacteria* were particularly attracted to 2-oxoglutarate. *Bacteroidetes*, *Alpha* and *Betaproteobacteria* seem to respond to alanine, with the later associating additionally with erythronic acid. *Chloroflexi* associate more with unknown compounds and a xylitol-like compound. In many cases, although a root exudate is produced by all plant species, the different bacterial groups have plant specific preferences.

Figure 27 – Heatmap for each major bacterial phylum (and proteobacterial classes) reflecting the number of positive and significant relationships (correlations) between a bacterial OTU and a specific root exudate per plant species. For clarity, exudates where it was not possible to calculate correlation with OTUs (represented with NA) from 3 or more plant species were removed from the graph. Ae – *Arrenatherum elatius*; Am – *Achillea milefollium*; Ap – *Alopecurus pratensis*; Dg – *Dactilys glomerata*; Pl – *Plantago lanceolata*; Ra – *Ranunculus acris*.

4.2 – Discussion

4.2.1 – Phytometer plants can be used as proxies for naturally grown plants

The rhizosphere bacterial communities on phytometer plants developed over time to be undistinguishable from the ones associated with natural individuals, which suggests a big influence of soil over plant origin. Furthermore, the natural specimens collected were bigger plants with more developed root systems when compared to the phytometer plants (own field observations), so the small variation is surprising and contrasts with previous reports which show a pronounced effect of plant development on the accompanying rhizosphere bacterial communities (Chaparro *et al.* 2014). These results reveal that with sufficient time for development and under the same environmental conditions, the rhizosphere bacterial communities evolve in the same direction, irrespectively of the history of the plant prior to the transplantation. These findings are in line with a recent study using the perennial herb *Boechera stricta*, transplanted into undisturbed meadows in Idaho. Bacteria associated with roots of greenhouse specimens had been largely replaced by bacteria present on the transplantation sites, becoming similar to indigenous plants and very dissimilar to greenhouse controls. The authors inferred that the root associated bacteria of these plants reached equilibrium with the surrounding soils at around 2 years after transplantation (Wagner *et al.* 2016). Taken together with the literature data, our findings open the possibility for a more controlled research on rhizosphere bacterial communities under natural settings, since plant individuals with defined age and developmental stage can be used as biological replicates to study rhizosphere bacterial communities under natural conditions.

4.2.2 – Factors shaping rhizosphere bacterial diversity

As generally reported, rhizosphere bacterial communities were distinct from bulk soil. Richness is higher in rhizosphere and this should relate to the fact that this study is based on RNA (16S rRNA) measurements, and rhizosphere bacteria are generally more active than in the corresponding bulk soil (Reinhold-Hurek *et al.* 2015). Studies based on DNA measurements found bacterial richness in the rhizosphere to be higher or similar to the accompanying bulk soil (Peiffer *et al.* 2013; Estendorfer *et al.* 2017). Bacterial community structure is distinct between soil compartments, with the major groups *Actinobacteria*, *Alphaproteobacteria*, *Bacteroidetes* and *Gammaproteobacteria* being enriched and *Acidobacteria*, *Planctomycetes*, *Chloroflexi*, *Deltaproteobacteria* and *Verrucomicrobia* being depleted in plant rhizospheres. Results are similar to studies of other plants, such as barley (Senga *et al.* 2017), lettuce (Schreiter *et al.* 2014), oilseed rape (Gkarmiri *et al.* 2017) and thale cress (*Arabidopsis thaliana*) (Bulgarelli *et al.* 2012), with the exception of *Actinobacteria* which are

sometimes found to be depleted in the rhizosphere compartment. Despite the differences observed in the abundant bacterial groups, it was interesting to observe that rare taxa are the ones most affected by the rhizosphere effect. The phyla most positively influenced are *Deinococcus-Thermus*. These bacteria are known for their resistance to extreme stresses and are reported in soils worldwide (Rosenberg 2014) but also associated with the rhizosphere (Lai *et al.* 2006). Nevertheless, their ecological role is not yet well explored due to a shortage in culturable representatives. Recently, Dawson and colleagues identified both these groups as enriched in the rhizosphere of 19 herbaceous grassland plants, including *Plantago lanceolata* and *Dactylis glomerata* (Dawson *et al.* 2017).

Numerous studies have shown that bacteria display a non-random environmental distribution in soils and communities can become increasingly different as the geographical distance between them increases, which can be due to environmental conditions at play and dispersal limitation (Martiny *et al.* 2006; Bell 2010; Nemergut *et al.* 2011). Rhizosphere bacterial community structure was distinct between the Biodiversity Exploratories locations, but this was not due to the geographic separation. The dissimilarity among bacterial community compositions likely matches the disparity in soils, with Schorfheide-Chorin being a young glacial landscape harbouring mainly histosol soils, Heinich consisting of calcareous bedrock predominantly harbouring cambisols and Swabian Alb consisting of calcareous bedrock with karst phenomena predominantly harbouring leptosols (Fischer *et al.* 2010). Most of the differences between the locations were due to abundance shifts of OTUs in three bacterial phyla (and proteobacterial classes) *Actinobacteria*, *Alphaproteobacteria* and *Firmicutes*. The *Clostridia* class of *Firmicutes,* known to be mostly composed of anaerobic or facultative anaerobic bacteria, are particularly abundant in Schorfheide-Chorin. This region is characterized by a higher soil water content and higher mean annual precipitation (Fischer *et al.* 2010) when compared to the other regions, representing in fact fen areas converted to grasslands (Klaus *et al.* 2016). A high level of water in soils leads to permanent anoxic areas which could foment the growth of these particular taxa (Lin *et al.* 2012; Concheri *et al.* 2017).

Even within each region or within soil type, the environmental filtering provided by soil properties has a big influence on the structure of rhizosphere bacterial communities, much larger than the influence of plants. In fact, the small influence of plants seems to be not even direct (e.g. difference in root architecture), but only modulated through root exudation. This is a surprising finding, since it is generally accepted that both plant species and soil type influence the rhizosphere bacteria (Lareen *et al.* 2016). Nevertheless, similar findings were obtained for two arcto-alpine plant species, *Oxyria dignya* and *Saxifraga oppositifolia*, investigated in three distinct regions. Although plant differences

were observed for endosphere bacterial communities, the rhizosphere of both plants was similar (Kumar *et al.* 2017). Also, studies on the root microbiota of the model plant *Arabidopsis thaliana* and close relatives, in their natural environment, revealed that rhizosphere communities are more strongly defined by environment than by host species (Schlaeppi *et al.* 2014). A possible reason for these findings relates to the close proximity existing between herbaceous plants in natural fields. In natural environments (and as we verified in our sampling areas), plants live in tight communities, their roots intermingle and occupy roughly the same soil depth. In this scenario, rhizosphere areas overlap between different plant species, which should lead to the development of highly similar bacterial communities. This also justifies the fact that, even though root exudate composition differs between plant species, it only has a small effect on bacteria present in the different plant rhizospheres, since an overlap in rhizosphere areas would ensure a relatively homogeneous distribution of the exuded compounds. This is even more likely, since the compounds evaluated are mostly part of plants primary metabolism and the differences observed in these are not qualitative but arise from differences in amounts of compounds exuded. Recent mesocosm studies observe an increase in microbial biomass as a result of increase of root exudate amounts (Eisenhauer *et al.* 2017) and diversity (Steinauer *et al.* 2016), but still studies are required to link the differential plant root exudate composition to alterations in rhizosphere bacterial community structures.

4.2.3 – A small number of OTUs are either specific or shared between plant rhizospheres

Considering the striking similarity of the bacterial communities amongst the different plant species, it was not surprising to detect bacterial OTUs consistently present in all rhizosphere environments. Though a small fraction (0.016%) of all OTUs detected, the ubiquitous OTUs constitute a major portion of the active rhizosphere communities, on average 58.3% of all sequenced reads, and mostly reflect members of *Actinobacteria* and *Alphaproteobacteria*. The OTUs which contribute the most are known to be associated with plant roots, having plant growth promoting abilities. Both *Bacillus* and *Streptomyces* can produce plant growth promoting molecules and are known for their capacity of producing antibiotics, antifungals and other molecules which serve as biocontrols for plant pathogens in the rhizosphere environment (Chen *et al.* 2008; Chung *et al.* 2008; Kumar *et al.* 2012; Cordovez *et al.* 2015; Viaene *et al.* 2016). Other important bacteria include the actinobacterial genera *Microlunatus*, *Pseudonocardia*, *Nakamurella* and *Microbacterium*, and although reported in plant rhizospheres, their ecological role in this environment is less clear. Nevertheless, the species *Microlunatus phosphovorus* is capable of polyphosphate accumulation, for which glucose can induce a release (Kawakoshi *et al.* 2012). This could constitute a source of phosphate for the P-limited plants, in exchange for C-rich compounds such as glucose. Apart from the two major groups

mentioned, also a high prevalence of *Planctomycetes* (75 OTUs) was found. This bacterial group is not so commonly reported as part of plant rhizospheres, though it has been found enriched in *Phragmites australis* (Zhang *et al.* 2013), cotton (Qiao *et al.* 2017) and *Caragana spp.* (Na *et al.* 2017). Moreover, a recent study of tundra soils dominated by lichens and of acidic peatlands has not only revealed a rich diversity of these bacteria, but also uncovered the potential of two isolated strains to degrade several polysaccharides, which reflect their potential to recycle plant debris (Ivanova *et al.* 2016).

A considerable fraction of all OTUs (22.6%) was plant species specific but these were only present in a minority of the plant individuals and are therefore likely governed by factors other than plant species. Nevertheless, when looking at differences in relative abundance, significant effects of plant species on specific OTUs were detected. Both positive or negative responses to a particular plant species, when compared to all others, were mostly reflected on rare OTUs, which shows the possible important role that these bacteria have in the rhizosphere environment. This is a relatively new finding in rhizosphere research (Jousset *et al.* 2017), only possible due to deep sequencing (here of about 1 million sequences per sample) which can cover the rich bacterial inventory present in this soil compartment. Recently, a study on the *Avena fatua* rhizosphere revealed that less abundant taxa were often keystone organisms on occurrence networks, suggesting an important role for these organisms in maintaining the structure of rhizosphere microbial communities (Shi *et al.* 2016). This importance of the rare bacteria in plant rhizospheres was also reported for maize (Dohrmann *et al.* 2013), tobacco (Saleem *et al.* 2016) and 19 distinct herbaceous species (Dawson *et al.* 2017). The present study suggests that, these rare taxa not only are present, but are in fact active in plant rhizospheres and are plant specific.

4.2.4 – Bacteria are selectively associated with specific root exudates

The impact of root exudates on bacterial communities is usually inferred from mesocosm experiments done under artificial conditions, implementing in many cases artificial root exudate solutions, which are very simplistic and diverge from the concentrations found in natural settings (usually much higher). The results reported in this study, though merely correlative, are a first step in understanding the relationship between root exudates and rhizosphere bacterial in natural settings.

The different plant species here studied exuded similar sets of compounds into the rhizosphere. Nevertheless, when evaluating the positive correlations between the amounts of compounds and the relative abundances of rhizosphere enriched bacteria, distinct bacterial groups were found to have different preferences. Sugars in general were not preferred by distinct bacterial taxa and were

utilized by all bacterial groups, with the exception of fructose, which seems to be preferred by *Chloroflexi*, *Firmicutes* and *Gammaproteobacteria*. These observations are consistent with previous work, where fewer impact of sugars in soil bacterial communities were reported compared to organic acids. This may be due to a general capability of bacteria for using sugars while organic acids are used by specialists (Eilers *et al.* 2010; Shi *et al.* 2011). *Gammaproteobacteria*, known rhizosphere colonizers (Espinosa-Urgel *et al.* 2002), showed a preference for 2-oxoglutarate (Figure 27), when compared to other bacterial groups. This preference may be related to the ability of many members of this class to fix nitrogen, as it has been shown that 2-oxoglutarate counteracts the repressive effect of the protein NifL on the *nif* transcriptional activator NifA of *Azotobacter vinelandii*, therefore enhancing nitrogen fixation (Little & Dixon 2003). *Chloroflexi* on the other hand have preference for a xylitol-like compound, which is basically restricted to this group. Xylitol is a molecule known for its bacteriostatic properties against bacteria, such as *Streptococcus* spp. (Kontiokari *et al*. 1995), so it is not surprising that bacteria are generally not associated with this compound. Nevertheless, *Chloroflexi* are known for their broad metabolic capabilities, and include described members which can grow in xylitol as a sole carbon source (Botero *et al.* 2004).

The present study also suggests some degree of plant specificity regarding individual compounds. In some cases, bacteria seem to prefer a compound originating from a particular plant species, even if this compound is also present in other plants. From these results it seems unlikely that this is related to a lack of competition for this compound in a particular plant species, since bacterial communities are similarly rich between plant rhizospheres. It is possible that these findings reflect not an attraction of bacteria to individual compounds, but to the synergistic effects of root exudates (and their amounts), selecting areas with available compound assortments which can satisfactorily cover most of their nutritional requirements. This work highlights the intricacies of rhizosphere plant-microbiome interactions and reflects the need for further, deepened investigations regarding root exudation effects on rhizosphere bacteria.

4.3 – References

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4.4 – Supplementary information

Supplementary Table 1 – List of identified polar metabolites of the Gas chromatography coupled mass spectrometry approach (GC-MS). Numbers in parenthesis show the mass of quantification (m/z). RT denotes the corresponding retention index.

compound	metabolite class	compound	metabolite class	compound	metabolite class	compound	metabolite class	compound	metabolite class
2-Aminoadipate (260)	organic acid	Threonine (218)	aminoacid	unknown (191) RT717	unknown	unknown (344) RT1259	unknown	unknown (217) RT1751	unknown
2-Isopropylmalate (275)	organic acid	Tryptophan (202)	aminoacid	unknown (75) RT722	unknown	unknown (103) RT1263	unknown	unknown (204) RT1756	unknown
2-Oxoglutarate (129)	organic acid	Tyramine (174)	aminoacid	unknown (75) RT737	unknown	unknown (103) RT1264	unknown	unknown (283) RT1774	unknown
3-Caffeoyl-trans-Quinic acid (345)	phenylpropanoid	Tyrosine (218)	aminoacid	unknown (278) RT789	unknown	unknown (319) RT1268	unknown	unknown (219) RT1776	unknown
4-Aminobutanoate [GABA] (174)	organic acid	Uracil (241)	nucleic base	unknown (103) RT803	unknown	unknown (179) RT1272	unknown	unknown (204) RT1780	unknown
5-Indolecarboxylic acid (305)	organic acid	Valine (144)	aminoacid	unknown (75) RT824	unknown	unknown (204) RT1272	unknown	unknown (127) RT1780	unknown
Adenine (264)	nucleic base	Xylitol-like (307) RT 1100	alcohol	unknown (172) RT825	unknown	unknown (217) RT1272	unknown	unknown (204) RT1788	unknown
Adenosine (236)	nucleotide	Xylose (217)	sugar	unknown (243) RT830	unknown	unknown (71) RT1278	unknown	unknown (216) RT1788	unknown
Adipic acid (111)	organic acid	unknown sugar (204) RT1781	sugar	unknown (229) RT838	unknown	unknown (319) RT1286	unknown	unknown (261) RT1791	unknown
Alanine (116)	aminoacid	unknown sugar (204) RT1913	sugar	unknown (237) RT851	unknown	unknown (273) RT1289	unknown	unknown (204) RT1796	unknown
Aminomalonic acid (218)	organic acid	unknown sugar (319) RT1314	sugar	unknown (179) RT867	unknown	unknown (273) RT1297	unknown	unknown (204) RT1798	unknown
Arginine (157)	amino acid	unknown sugar (319) RT1321	sugar	unknown (191) RT908	unknown	unknown (299) RT1298	unknown	unknown (117) RT1803	unknown
Asparagine (231)	amino acid	unkown compound (204) RT1517	unknown	unknown (306) RT913	unknown	unknown (132) RT1300	unknown	unknown (173) RT1809	unknown
Aspartate (232)	amino acid	unkown compound (357) RT1526	unknown	unknown (174) RT924	unknown	unknown (299) RT1309	unknown	unknown (191) RT1825	unknown
Azelaic acid (317)	organic acid	unkown fatty acid (339) RT1518	lipid	unknown (263) RT932	unknown	unknown (285) RT1310	unknown	unknown (204) RT1834	unknown
Benzaldehyde (257)	aldehyde	unknown (174) RT433	unknown	unknown (71) RT934	unknown	unknown (57) RT1314	unknown	unknown (204) RT1853	unknown
Benzoic acid (267)	organic acid	unknown (191) RT435	unknown	unknown (120) RT940	unknown	unknown (71) RT1316	unknown	unknown (319) RT1860	unknown
beta-Alanine (248)	aminoacid	unknown (87) RT435	unknown	unknown (158) RT944	unknown	unknown (319) RT1316	unknown	unknown (356) RT1864	unknown
Butylamine (202)	amin	unknown (75) RT435	unknown	unknown (227) RT945	unknown	unknown (160) RT1319	unknown	unknown (306) RT1865	unknown
cis-Aconitate (229)	organic acid	unknown (207) RT436	unknown	unknown (174) RT956	unknown	unknown (333) RT1325	unknown	unknown (217) RT1877	unknown
Coumaric acid (308)	organic acid	unknown (89) RT439	unknown	unknown (217) RT964	unknown	unknown (174) RT1327	unknown	unknown (204) RT1902	unknown
Erythronic acid (292)	organic acid	unknown (89) RT440	unknown	unknown (142) RT973	unknown	unknown (361) RT1330	unknown	unknown (259) RT1906	unknown
Fructose (217)	sugar	unknown (117) RT443	unknown	unknown (103) RT974	unknown	unknown (318) RT1334	unknown	unknown (160) RT1909	unknown
Gluconate (333)	organic acid	unknown (173) RT443	unknown	unknown (103) RT982	unknown	unknown (204) RT1340	unknown	unknown (361) RT1909	unknown
Glucose-6-phosphate (387)	sugar	unknown (112) RT451	unknown	unknown (223) RT983	unknown	unknown (205) RT1347	unknown	unknown (204) RT1914	unknown
Glutamate (246)	aminoacid	unknown (207) RT454	unknown	unknown (245) RT987	unknown	unknown (293) RT1357	unknown	unknown (361) RT1919	unknown
Glutamine (155)	aminoacid	unknown (117) RT459	unknown	unknown (342) RT996	unknown	unknown (311) RT1362	unknown	unknown (259) RT1920	unknown
Glycerol 3-phosphate (357)	lipid	unknown (77) RT466	unknown	unknown (117) RT1034	unknown	unknown (155) RT1366	unknown	unknown (205) RT1926	unknown
Homoserine (218)	aminoacid	unknown (127) RT470	unknown	unknown (117) RT1037	unknown	unknown (297) RT1372	unknown	unknown (361) RT1926	unknown
Isoleucine (158)	aminoacid	unknown (58) RT470	unknown	unknown (245) RT1049	unknown	unknown (335) RT1385	unknown	unknown (217) RT1936	unknown
Lactic acid (191)	organic acid	unknown (188) RT470	unknown	unknown (103) RT1055	unknown	unknown (331) RT1441	unknown	unknown (223) RT1937	unknown
Lactose (361)	sugar	unknown (116) RT474	unknown	unknown (245) RT1065	unknown	unknown (217) RT1443	unknown	unknown (204) RT1939	unknown

Leucine (158)	aminoacid	unknown (258) RT494	unknown	unknown (277) RT1082	unknown	unknown (331) RT1443	unknown	unknown (361) RT1948	unknown
Lysine (156)	aminoacid	unknown (125) RT508	unknown	unknown (117) RT1121	unknown	unknown (324) RT1456	unknown	unknown (204) RT1958	unknown
Melibiose (361)	sugar	unknown (355) RT515	unknown	unknown (217) RT1123	unknown	unknown (327) RT1456	unknown	unknown (319) RT1968	unknown
Methionine (176)	aminoacid	unknown (117) RT529	unknown	unknown (57) RT1127	unknown	unknown (319) RT1465	unknown	unknown (204) RT1971	unknown
Myo-Inosito1-phosphate (318)	sugar	unknown (201) RT532	unknown	unknown (93) RT1132	unknown	unknown (319) RT1466	unknown	unknown (83) RT1974	unknown
myo-Inositol (305)	alcohol	unknown (158) RT533	unknown	unknown (103) RT1133	unknown	unknown (319) RT1487	unknown	unknown (362) RT1978	unknown
N-Acetylglucosamine (156)	sugar	unknown (281) RT548	unknown	unknown (69) RT1140	unknown	unknown (167) RT1487	unknown	unknown (204) RT1986	unknown
Noradrenalin (174)	alcaloid	unknown (241) RT553	unknown	unknown (174) RT1143	unknown	unknown (128) RT1504	unknown	unknown (525) RT1992	unknown
Octadecadienoic acid (337)	lipid	unknown (89) RT575	unknown	unknown (69) RT1150	unknown	unknown (185) RT1512	unknown	unknown (201) RT1994	unknown
Octadecatrienoic acid (335)	lipid	unknown (169) RT581	unknown	unknown (217) RT1155	unknown	unknown (117) RT1532	unknown	unknown (217) RT1994	unknown
Octadecenoic acid (339)	lipid	unknown (288) RT590	unknown	unknown (103) RT1166	unknown	unknown (204) RT1603	unknown	unknown (361) RT2003	unknown
Ornithine / Citrullin (142)	aminoacid	unknown (169) RT592	unknown	unknown (217) RT1166	unknown	unknown (167) RT1609	unknown	unknown (204) RT2011	unknown
Phenylalanine (192)	aminoacid	unknown (117) RT605	unknown	unknown (292) RT1166	unknown	unknown (204) RT1615	unknown	unknown (119) RT2019	unknown
Phosphoenolpyruvate (247)	sugar	unknown (281) RT610	unknown	unknown (295) RT1171	unknown	unknown (197) RT1637	unknown	unknown (297) RT2022	unknown
Pinitol (260)	alcohol	unknown (219) RT616	unknown	unknown (69) RT1171	unknown	unknown (239) RT1643	unknown	unknown (217) RT2026	unknown
Proline (142)	aminoacid	unknown (179) RT621	unknown	unknown (57) RT1173	unknown	unknown (83) RT1643	unknown	unknown (361) RT2053	unknown
Racr_spec unknown (306) RT832	unknown	unknown (284) RT632	unknown	unknown (217) RT1180	unknown	unknown (255) RT1654	unknown	unknown (91) RT2055	unknown
Rhamnose (117)	sugar	unknown (74) RT632	unknown	unknown (292) RT1203	unknown	unknown (204) RT1655	unknown	unknown (327) RT2065	unknown
Ribose (217)	sugar	unknown (186) RT642	unknown	unknown (174) RT1212	unknown	unknown (57) RT1655	unknown	unknown (204) RT2068	unknown
Salicylic acid (267)	organic acid	unknown (192) RT643	unknown	unknown (285) RT1213	unknown	unknown (259) RT1657	unknown	unknown (362) RT2071	unknown
scyllo-inositol (204)	alcohol	unknown (158) RT649	unknown	unknown (75) RT1218	unknown	unknown (91) RT1660	unknown	unknown (91) RT2097	unknown
Serine (204)	amino acid	unknown (159) RT650	unknown	unknown (103) RT1218	unknown	unknown (56) RT1675	unknown	unknown (57) RT2144	unknown
Shikimate (204)	organic acid	unknown (205) RT653	unknown	unknown (156) RT1226	unknown	unknown (260) RT1676	unknown	unknown (204) RT2147	unknown
Sorbitol like (217)	alcohol	unknown (173) RT665	unknown	unknown (149) RT1233	unknown	unknown (204) RT1683	unknown	unknown (153) RT2157	unknown
Succinate-like (147)	organic acid	unknown (280) RT669	unknown	unknown (160) RT1240	unknown	unknown (82) RT1700	unknown	unknown (191) RT2175	unknown
Sucrose like (361)	sugar	unknown (126) RT670	unknown	unknown (103) RT1243	unknown	unknown (204) RT1731	unknown	unknown (204) RT2224	unknown
Tartaric acid (292)	organic acid	unknown (75) RT690	unknown	unknown (71) RT1257	unknown	unknown (149) RT1745	unknown	unknown (204) RT2329	unknown
Threitol (217)	alcohol	unknown (89) RT706	unknown	unknown (295) RT1259	unknown	unknown (204) RT1749	unknown		

Supplementary Table 2 – List of plant parameters measured for each plant.

Plant parameters	Units
Plant species	*Arrenatherum elatius*; *Achillea millefolium*; *Alopecurus pratensis*; *Dactilys glomerata*; *Plantago lanceolata*; *Ranunculus acris*
Plant growth form	herb or grass
Root fresh mass	g
Plant fresh mass	g
Root dry matter content	mg/g
Root carbon content	%
Root nitrogen content	%

Supplementary Table 3 – List of plant parameters measured for each plant.

Soil parameters	Units
Exploratory location	Swäbische-Alb; Heinich-Dün; Schorfheide-Chorin
Land Use Intensity Index (LUI)	*
pH	0-14
Soil carbon content	gkg^{-1}
Soil nitrogen content	gkg^{-1}

*****See Blüthgen *et al.*, 2012

Supplementary Table 4 - List of identified polar metabolites of the Gas chromatography coupled mass spectrometry approach (GC-MS) that were selected by the forward selection process for each soil type. Numbers in parenthesis show the mass of quantification (m/z). RT denotes the corresponding retention index. For Stagnosol no compounds were selected by forward selection.

Vertisol	Luvisol	Cambisol	Histosol	Leptosol	Stagnosol
Adipic acid (111)	2-Oxoglutarate (129)	Cis-Aconitate (229)	Adipic acid (111)	Unknown sugar (319) RT1321	
Sorbitol like (217)	Unknown (169) RT581	Aspartate (232)	Unknown (306) RT913	Unknown (82) RT1700	
Unknown (142) RT973	Unknown (241) RT553	Unknown (69) RT1140		Unknown (237) RT851	
	Unknown (319) RT1286	Unknown (132) RT1300		Unknown (299) RT1309	
		Unknown (156) RT1226		Unknown (333) RT1325	

Chapter 5 – Dynamics of bacterial colonisation of minerals in soils

5.1 – Results

This chapter attempts to evaluate the bacterial colonisation of mineral surfaces in soils. To do so, containers with a mineral mixture were buried in different soils and the development of bacterial communities was monitored through time. Two distinct experiments were carried out in order to ascertain the importance of nutrient quality for the colonisation of the novel minerals. In the simple carbon addition experiment, known root exudate compounds were loaded onto the minerals, while in the complex carbon addition experiment, plant roots were mixed with the minerals. Further experimental details are given in Experimental Procedures (Chapter 3).

5.1.1 – High throughput sequence read statistics

Next-generation amplicon sequencing of the V3 region of the 16S rRNA gene was employed to determine the structure of the bacterial community per sample. After quality filtering, denoising and chimera removal, approximately 326,000,000 sequences were obtained. A subset of approximately 60,000,000 sequences could be assigned using QIIME closed reference approach against the SILVA SSU Ref 128 database (clustered at 99% sequence similarity) at 99% sequence identity and were used for subsequent analysis. Sequences assigned to chloroplast were removed and the remainder could be clustered in 28241 operational taxonomic units (OTUs). Near saturation was observed for the rarefaction curves performed for the OTUs present in control soil, adjacent soil, mineral and root samples, which indicated that our sequence inventory covered most of the taxa present in the samples (Figure 28). After calculation of rarefaction and alpha diversity metrics, counts were normalized.

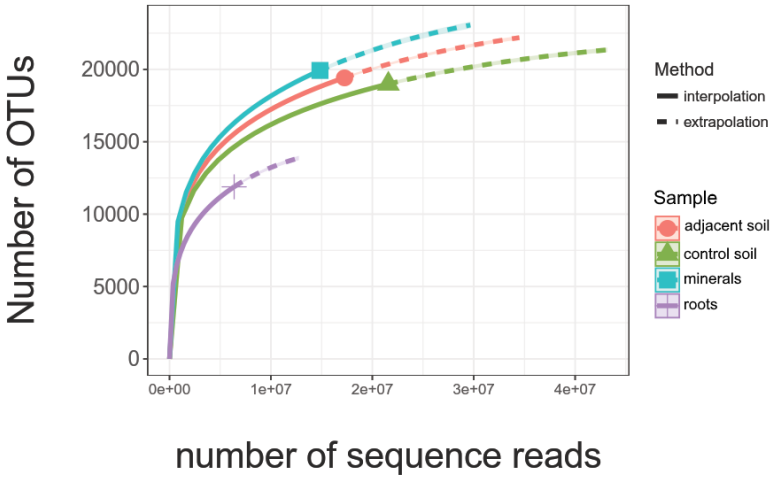

Figure 28 – Rarefaction curves for control soil, adjacent soil, mineral and root samples.

5.1.2 – Temporal development of bacterial communities on minerals and root litter

The alpha diversity of bacterial communities present in control soils, adjacent soils, minerals and roots was analysed. Concerning the simple carbon addition experiment (Figure 29), both richness and diversity were stable in control soils at first, but on day 119 there was a pronounced decrease in the two metrics followed by a recovery. Evenness remained constant. For the adjacent soil this drop in alpha diversity was only discernible for richness, while diversity and evenness remained constant over time. Bacterial communities on minerals revealed a high richness and diversity already before being introduced to the new environment (day 0). This was unexpected since the minerals are artificial and not expected to harbour a complex bacterial community. There was a drop in diversity and evenness in the first timepoint (day 14), which suggests big shifts in bacterial community composition. From the first timepoint on all the metrics increased, reaching stable values around 60 days. Richness values reached the ones for control and adjacent soil, but communities were less diverse.

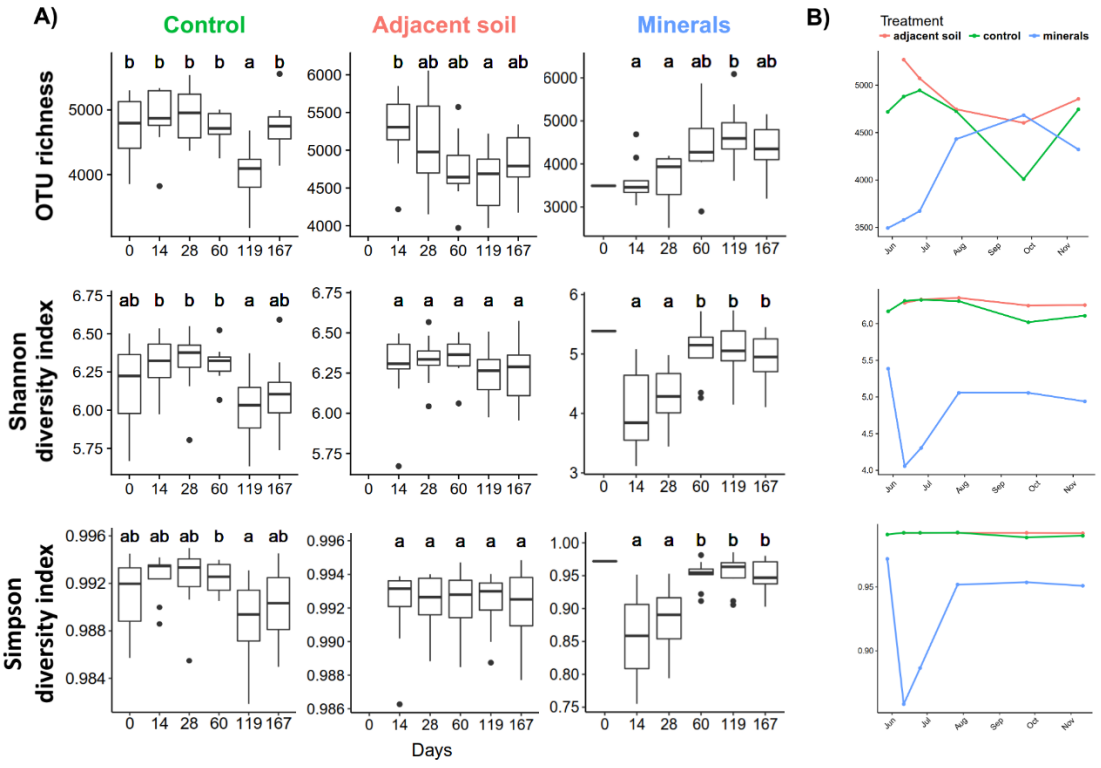

Figure 29 – Alpha diversity measures for bacterial communities at OTU level, for control, adjacent soil and minerals for the simple carbon addition experiment. A) Boxplots reflect alpha diversity metrics over the distinct timepoints. Letters on top reflect significant differences ($p<0.05$) as a result of multcomp test. B) Line graph with mean values of alpha diversity.

In the complex carbon addition experiment (Figure 30), control soils had high richness at the beginning followed by a significant drop at day 29. From this to day 64 (corresponding to winter period) there was no difference. At day 210 there was an increase in richness followed by a small decrease (again at the beginning of winter) which was not significant. Diversity and evenness followed the same trend, but the differences were mostly not significant. Bacterial communities in adjacent soil followed the same trend as control soils, but the changes were not significant. Both mineral and root material (again unexpectedly) harboured quite rich and diverse communities already at the beginning of the experiment. Like the simple carbon addition experiment, there was a drop in diversity and evenness in these communities (day 29), which again suggests substantial changes in bacterial community composition. From this point on there was an increase in diversity and evenness, peaking at day 210. Richness remained unchanged over time. On roots, there was a significant steady increase of richness and diversity over time, but the values were always lower than the ones on mineral material.

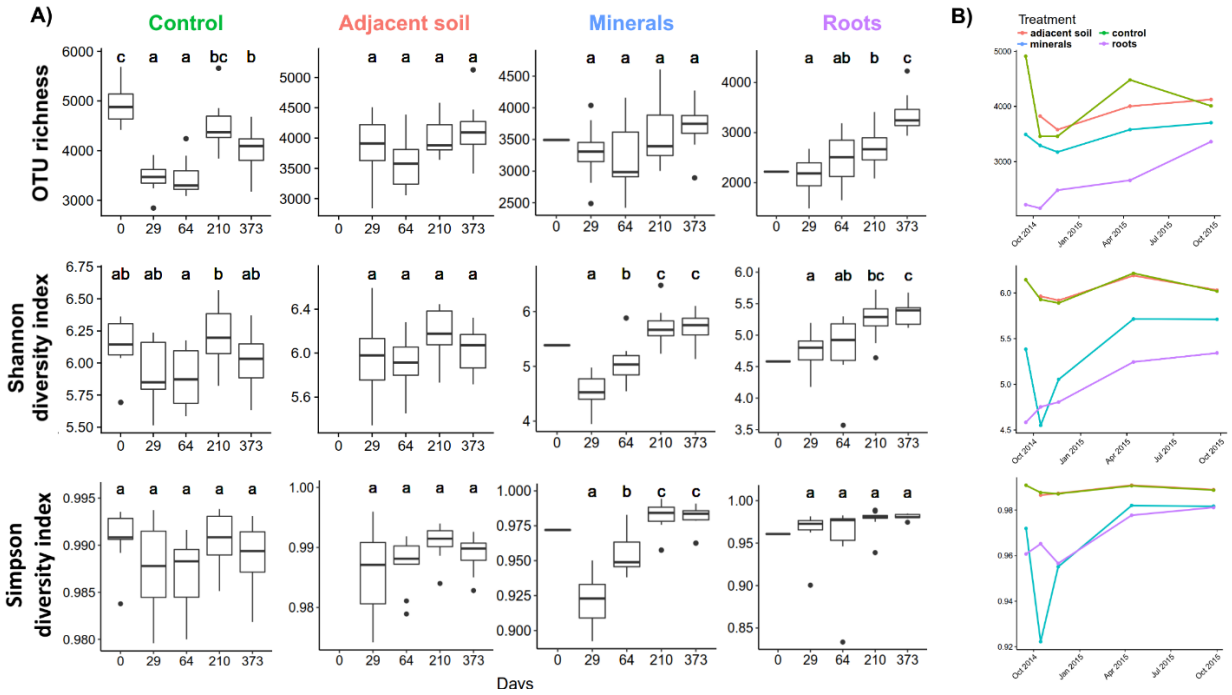

Figure 30 – Alpha diversity measures for bacterial communities at OTU level, for control, adjacent soil minerals and roots for the complex carbon addition experiment. A) Boxplots reflect alpha diversity metrics over the distinct timepoints. Letters on top reflect significant differences ($p<0.05$) as a result of multcomp test. B) Line graph with mean values of alpha diversity.

The change of bacterial numbers over time was also evaluated for the different samples using qPCR measurements on the bacterial 16S rRNA gene (Figure 31). The patterns were similar to the ones obtained for richness for both experiments, revealing that an increase in numbers over time was

generally accompanied with an increase of diversity. Roots represented an exception since there was an increase in diversity over time, but there was no significant increase in bacterial numbers (even though there seemed to be an increase).

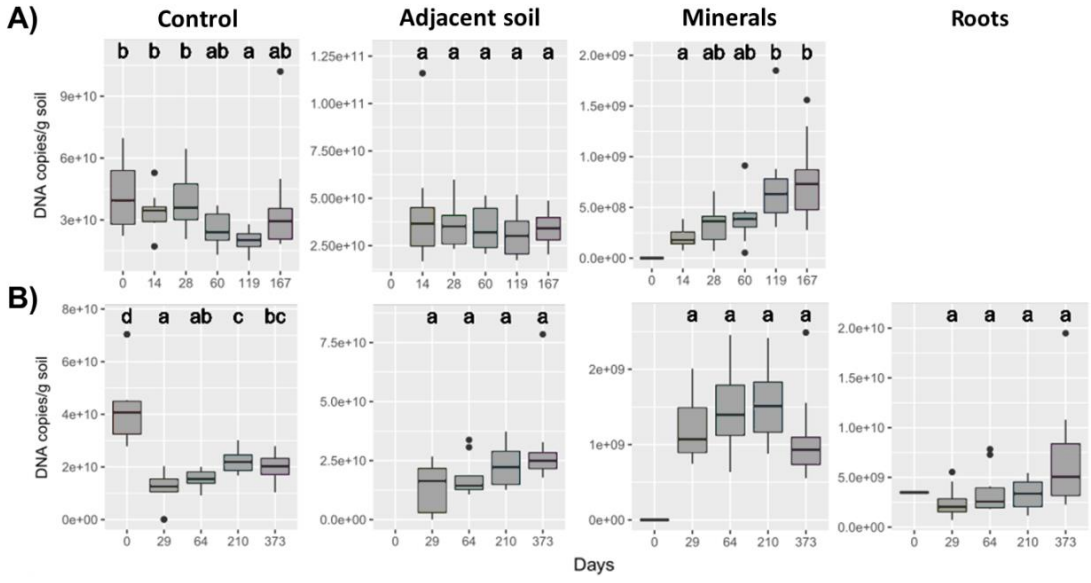

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Figure 31 – Boxplots of 16S rRNA gene copies per gram of soil over time for control, adjacent soil, minerals and roots for A) the simple carbon addition experiment or B) the complex carbon addition experiment. Letters on top reflect significant differences ($p<0.05$) as a result of multcomp test.

The determinants of bacterial community composition were evaluated using NMDS based on weighted UniFrac distances at the OTU level (Figure 32). First, control and adjacent soil bacterial communities for both experiments were very similar (PERMANOVA $p = 0.5904$) and were not altered through time (apart from 3 adjacent soil samples on the simple carbon addition experiment at timepoint 1 and 2, and control soil samples on the complex carbon addition experiment at timepoint 1). Second, the mineral communities of both experiments (simple ad complex carbon addition) were dramatically different from the ones on the respective material prior to burial. Third, a temporal progression was observed for minerals and root communities, making them increasingly similar with time. Both mineral communities, despite slight differences, develop in parallel with time.

It would be expected that distinct community structures would develop depending on the substrate complexity, especially on the initial stages of colonization, where simple carbon sources have not yet been depleted. The data obtained does not verify this hypothesis. Although bacterial communities in minerals of both experiments underwent a big shift when exposed to the new conditions, the resulting communities were quite similar. This response is also independent of seasonality since the simple carbon addition experiment was started when the complex carbon addition experiment was already in final stages.

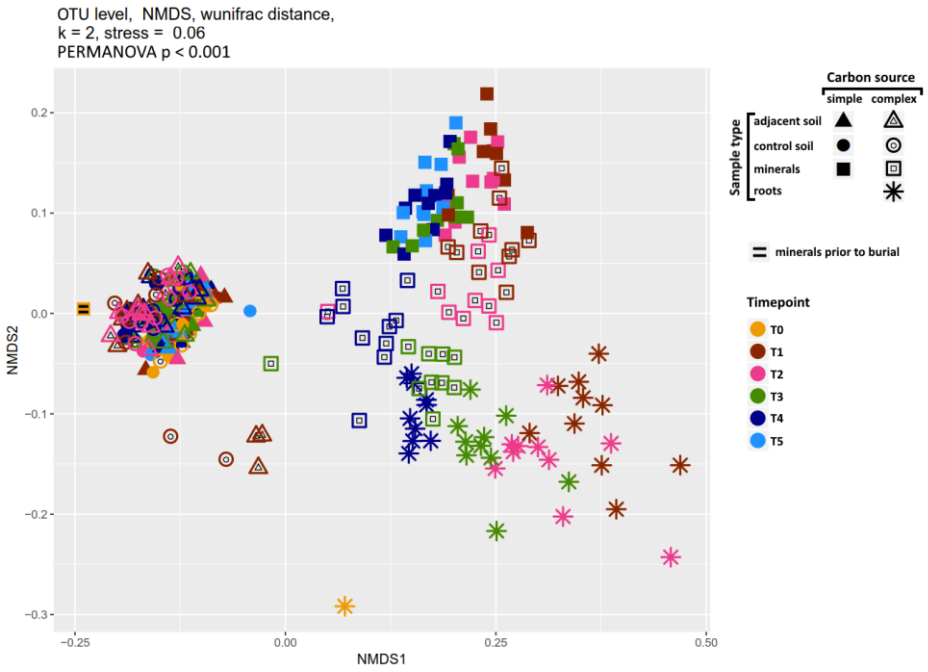

Figure 32 – NMDS plot for all samples. Community on naked minerals (T0) is the same for both experiments (striped square). Simple carbon addition experiment: T0 – 0 days; T1 – 14 days; T2 – 26 days; T3 – 60 days; T4 – 119 days; T5 – 167 days. Complex carbon addition experiment; T0 – 0 days; T1 – 29 days; T2 – 64 days; T3 – 210 days; T4 – 373 days.

The pairwise distances (weighted UniFrac) between bacterial communities on minerals of the simple and complex carbon addition experiments were compared to the pairwise distances between all minerals and the other compartments (adjacent soil, control soil and roots). Although bacterial communities were distinct between the simple and complex carbon addition experiments (PERMANOVA $p < 0.001$), it was clear that they were more similar between minerals of both experiments than to communities in any of the other compartments (Figure 33). This revealed that mineral properties are a major driver of bacterial communities, likely bigger than the expected effects of the distinct carbon compounds available in the environment.

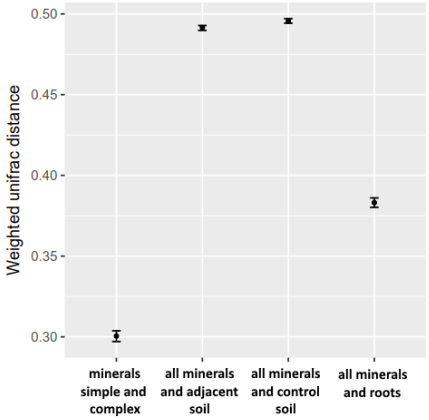

Figure 33 – Pairwise comparisons of distances (weigthed UniFrac) between bacterial communities on minerals of the simple and complex carbon addition experiments and of all minerals compared to the compartments adjacent soil, control soil and roots. Dots show the group-wise mean values, whiskers reflect 95% confidence intervals.

Bacterial communities on the simple carbon addition experiment seemed to change less through time as compared with the complex carbon addition experiment. In fact they appear to reach a state of equilibrium towards the end of the experiment. In contrast to the simple carbon addition experiment, communities of both minerals and roots of the complex carbon addition experiment were still developing and no equilibrium was reached (Figure 33). The influence of land use intensity (LUI) was also evaluated but it did not appear to have an effect on mineral or root bacterial communities (Figure 34).

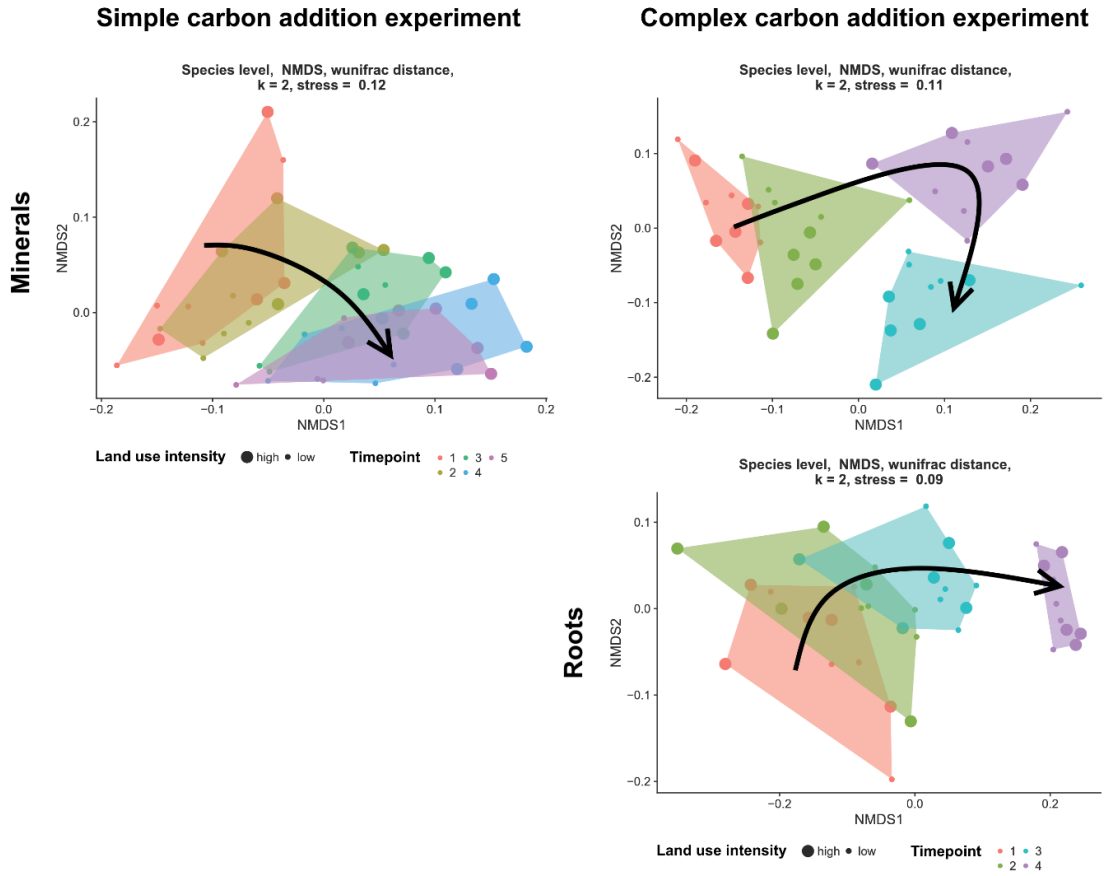

Figure 34 – NMDS plot for mineral and root samples for both experiments. Arrows depict the temporal development.

When looking at control and adjacent soil communities in more detail, we could observe that the change of the communities is smaller than the differences observed between plots. Furthermore, an effect of land use could be observed (Figure 35).

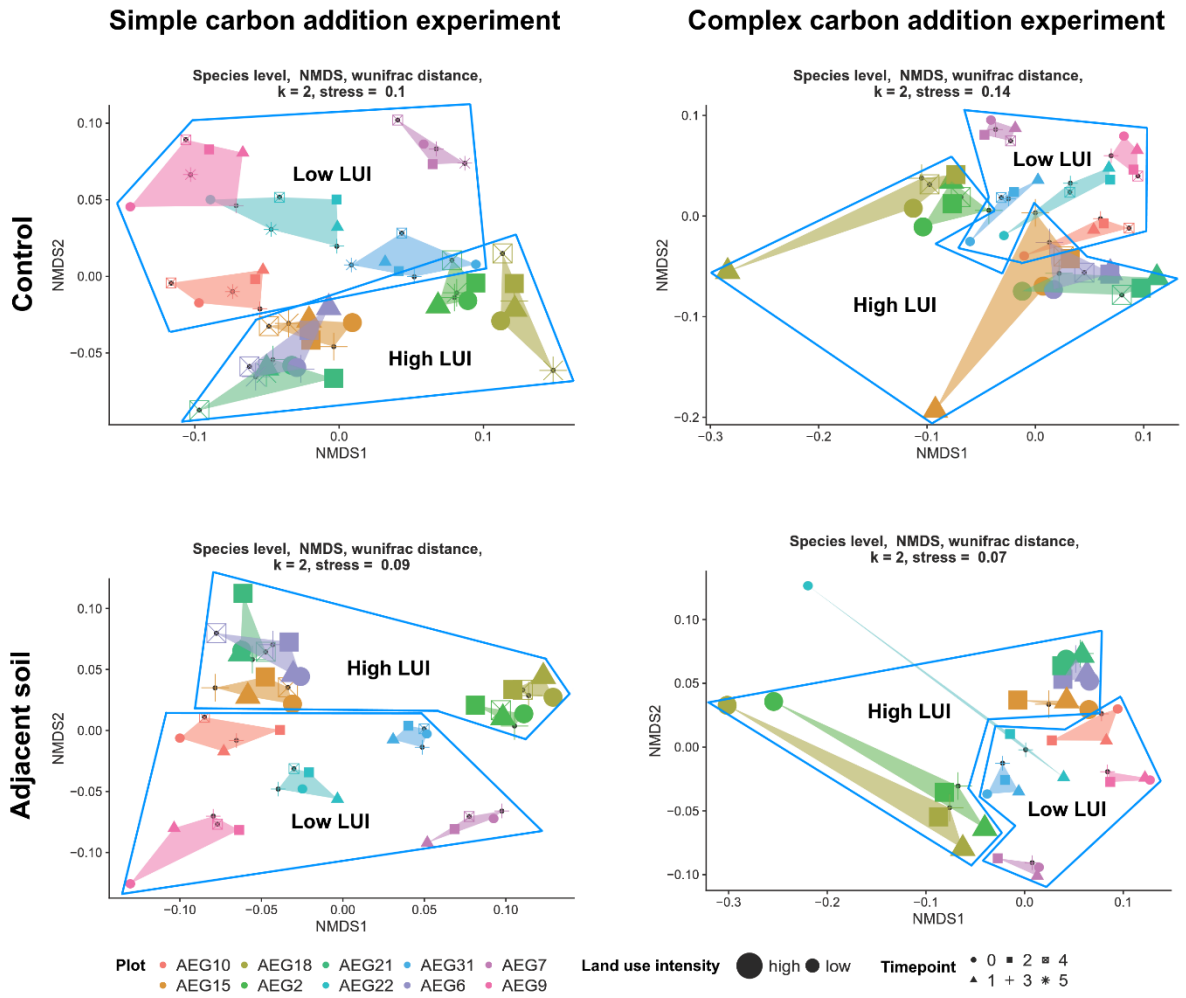

Figure 35 – NMDS plot for control and adjacent soil for both experiments grouped according to plot. Blue boxes group samples according to land use index (LUI).

5.1.3 – Distinct bacterial OTUs follow specific temporal patterns

In order to investigate the temporal changes on relative abundances of mineral and root bacterial communities, abundant OTUs were selected (relative abundance above 0.1% (Pedrós-Alió 2012; Lynch & Neufeld 2015)) (Table 7).

A total of 461 OTUs were selected: 64 were shared between all sample types, 130 were shared between minerals of both experiments, 58 were shared between minerals and roots of the complex carbon addition experiment, and 8 were shared between minerals of the simple carbon addition experiment and roots of the complex carbon addition experiment (Figure 9). Although only a minor fraction of the total OTU number (0.013% - 0.023%), these selected OTUs represent the major active fraction in terms of relative abundance (60.5% - 87.7%). Although some of these OTUs were abundant in the surrounding control soils, the majority was not (Table 6). For roots, 7 OTUs could not be detected in the corresponding controls.

Table 7 – Number of selected abundant OTUs, their mean relative abundance at different timepoints, percentage of all OTUs and their representation on control soil OTUs.

		Summed mean relative abundance (%)							Nº OTUs also
Sample type	**Nº of OTUs**	**T0**	**T1**	**T2**	**T3**	**T4**	**T5**	**% of all OTUs**	**abundant in control**
Experiment 1 minerals	229	69	75.5	74	67.7	68.2	70.4	0.013	104
Experiment 2 minerals	278	69.7	77.1	71.5	62	60.5	-	0.019	103
Exp2 roots	279	87.7	74.7	75.5	71.7	72	-	0.023	17

For each of the abundant OTUs a temporal activity pattern was estimated based on the changes in relative abundance through time. Because some OTUs are much more abundant than others, the relative abundance values were adjusted to a 0-100 scale from the smallest to the largest value. In the same way, to enable comparability between experiments, time was also adjusted to a 0-100 scale. The resulting patterns could be clustered into six major groups which reveal distinct activities (Figure 36). These activity patterns were not particular to one experimental setting or sample type, but present in both, minerals and roots.

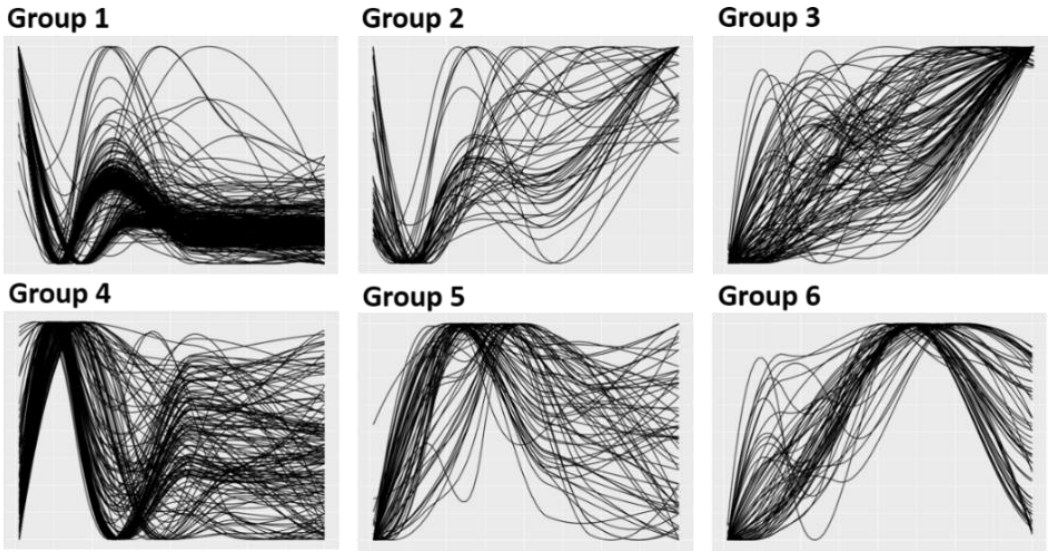

Figure 36 – Groups of OTUs with distinct activity patterns (N=461). Lines reflect shifts in relative abundance (y-axis, adjusted to a 0-100 scale) through time (x-axis, adjusted to a 0-100 scale), which are a result of smooth curve fitting using local polynomial regression (LOESS). See page 55 for explanation on the generation of the data.

Generally, the bacterial OTUs within a group were specific to the sample type and/or experiment. One exception were group 1 OTUs, which were shared between minerals. Also, many OTUs from group 4 were shared between all sample types, and especially between minerals and roots of the complex carbon addition experiment (Figure 37). These were also the most important group in terms of relative abundance, being dominant at all stages of both experiments (with exception of minerals and roots prior to burial). Its important to recall that a distinct OTU can be shared between both minerals and root compartments but might reveal a distinct temporal activity pattern only in one or two of the compartments. Therefore, the counts of the group Venn diagrams do not sum up to the Venn diagrams of all OTUs.

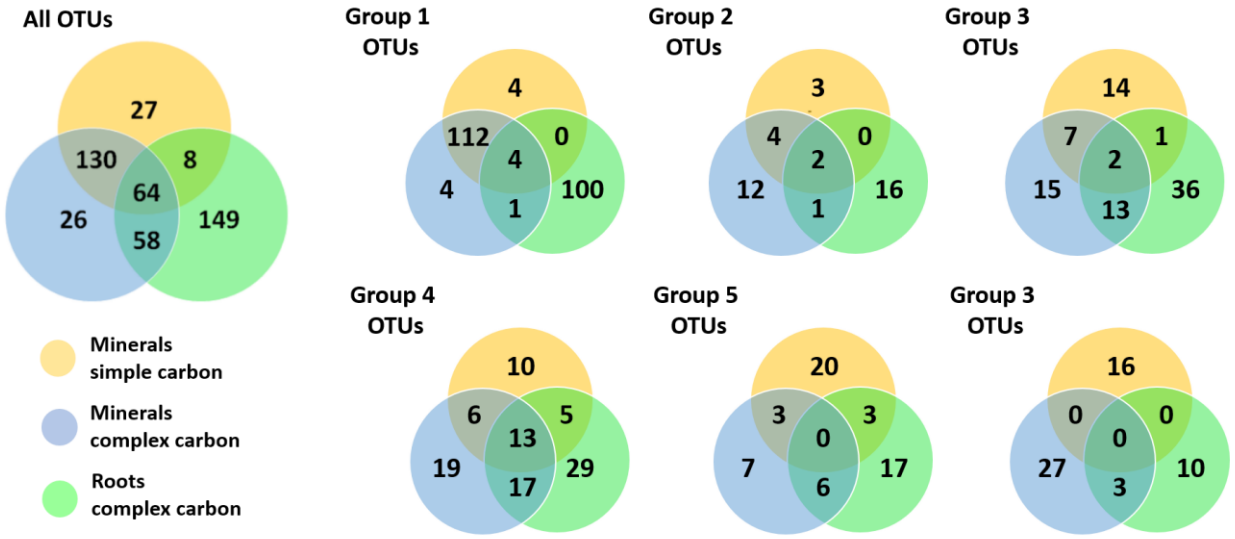

Figure 37 – Venn diagrams reflecting the number of bacterial OTUs shared between minerals and roots of both experiments, per group.

In order to evaluate whether the six activity patterns observed were governed by the same OTUs or groups known to the have the same functional roles, the taxonomic signal was investigated for each group. Most of the genera (59.6% of all taxa) were specific to one of the groups, with 52 OTUs occurring exclusively in group 1, 6 only in group 2, 11 only in group 3, 22 only in group 4, 4 only in group 5 and 7 only in group 6.

5.1.3.1 – Taxonomic characteristics of temporally divergent groups

Group 1 encompassed the bacterial OTUs which were the main colonizers of the minerals prior to burial (62.3% and 62%) and roots (78.7%) and which were disturbed when exposed to the new conditions in the soil (3.1% - 4.24% relative abundance). These OTUs quickly decreased in abundance and the majority became rare or were eradicated. The increase in relative abundance observed after the initial drop is an artefact created by the curve fitting method and should be disregarded (see examples in Supplementary Figure 1).

OTUs with this activity pattern were mostly the same for minerals of both experiments (112 OTUs), revealing minerals as a specific selective environment (Figure 38). These belonged mainly to unclassified genera of *Chthoniobacterales*, *Planctomycetaceae* and *Tepidisphaeraceae* but also to the nematode symbiont Candidatus *Xiphinematobacter*. Regarding the root compartment, OTUs of group 1 were distinct from the minerals and belonged mainly to *Tahibacter*, *Devosia* and to unclassified genera of *Fimbriimonadales*, *Microbacteriaceae*, *Xanthomonadales*, *Saccharibacteria* and *Rhodobacteriaceae*.

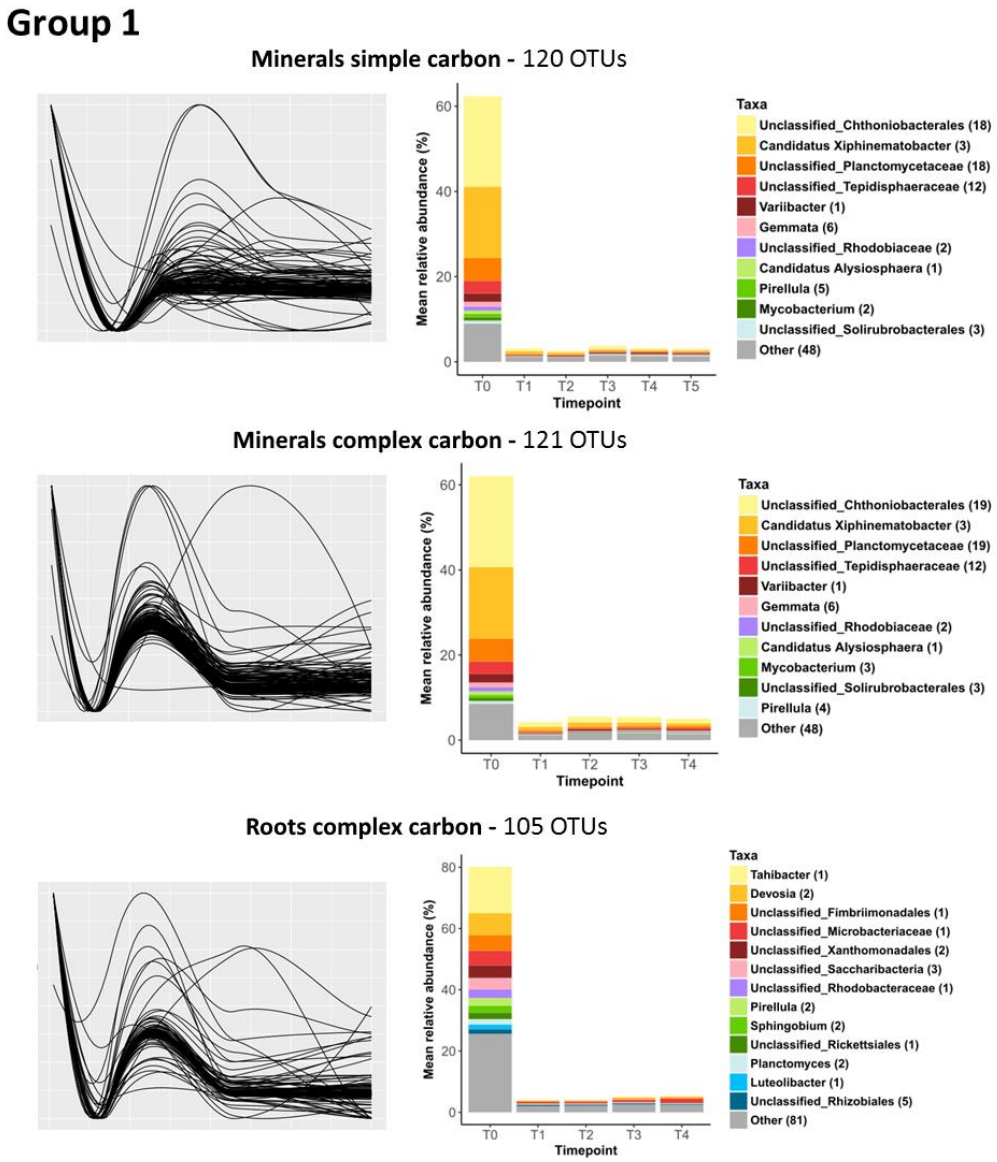

Figure 38 – OTUs which compose group 1 and their taxonomic affiliation at genus level, split according to experiment and compartment. Numbers in brackets represent how many OTUs represent the depicted genera.

Group 2 reflected the OTUs which were also abundant in the minerals and roots prior to burial and affected once in the new environment, but these were able to recover over time becoming as abundant or even surpassing the values at beginning of the experiment (Figure 39). These OTUs were not many and were mostly specific for each compartment. Nevertheless, more OTUs were shared between minerals of both experiments (4 OTUs) than between minerals and roots of the complex carbon addition experiment (1 OTUs). One OTU belonging to *Bradyrhizobium* was especially important in the minerals of both experiments, contributing the most of all OTUs in all timepoints.

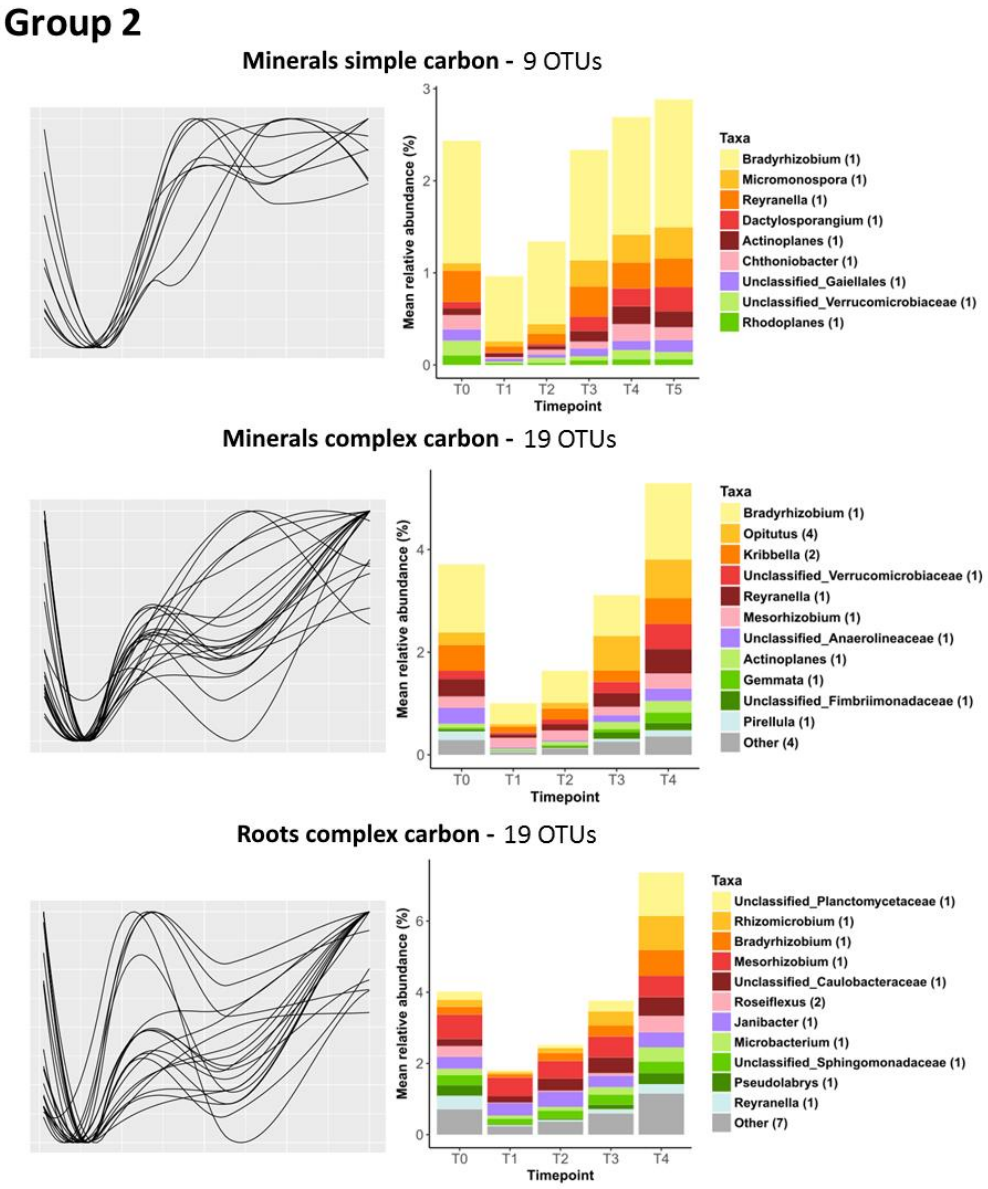

Figure 39 – OTUs which compose group 2 and their taxonomic affiliation at genus level, split according to experiment and compartment. Numbers in brackets represent how many OTUs represent the depicted genera.

Groups 3 was comprised of OTUs which were not present or rare in the minerals and roots prior to burial, but had a gradual increase as the experiment progressed, and despite some having a hiatus in the middle, on the last measurement they registered the highest abundance value since the start of the experiment. OTUs with this activity pattern were most important for the roots of experiment 2, making 31.3% in relative abundance (Figure 40). From this group, more OTUs were shared between compartments of the complex carbon addition experiment (13 OTUs) than between the minerals of

both experiments (7 OTUs). OTUs of *Ramlibacter* and *Nocardioides* seemed most important for minerals of the simple carbon addition experiment and were not found in roots.

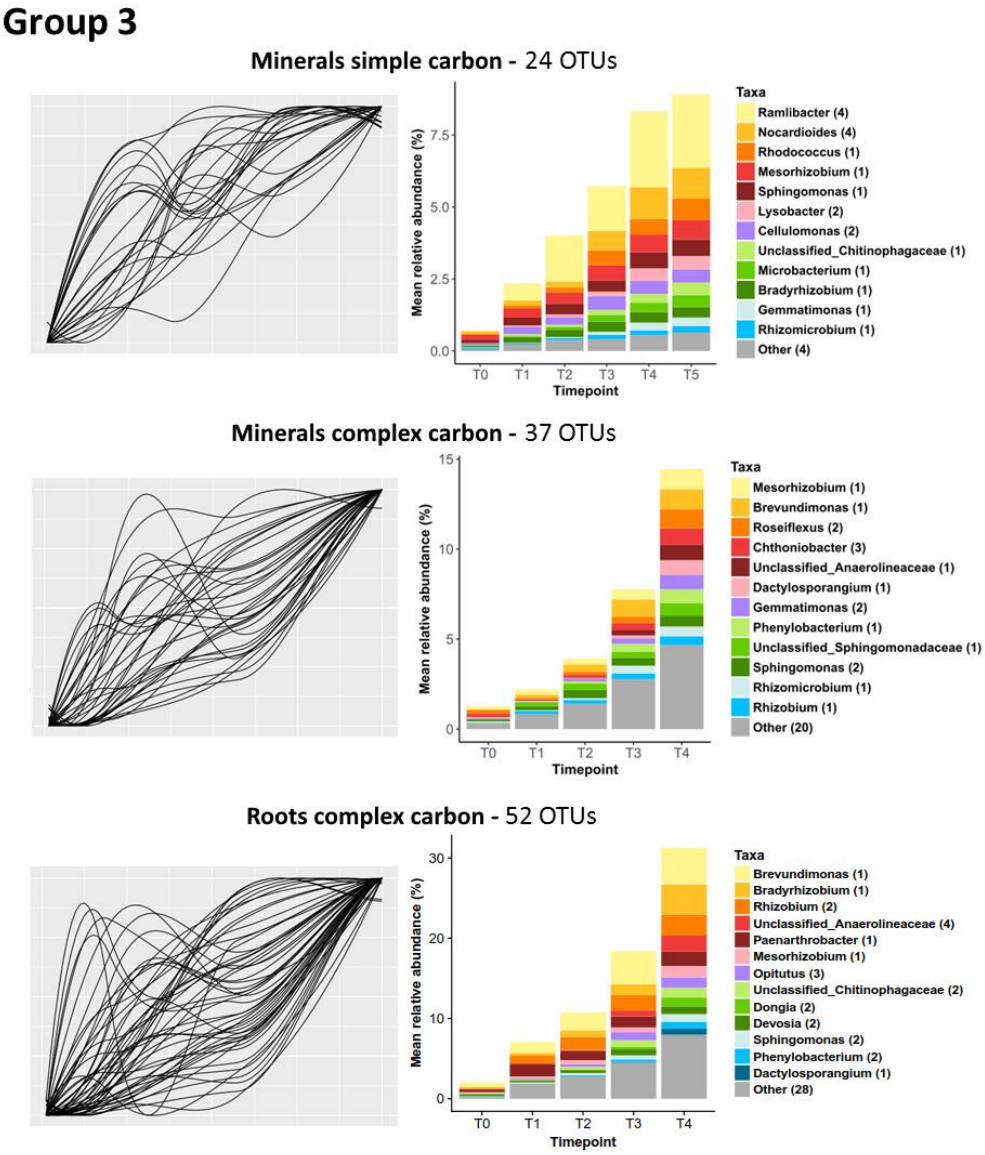

Figure 40 – OTUs which compose group 3 and their taxonomic affiliation at genus level, split according to experiment and compartment. Numbers in brackets represent how many OTUs represent the depicted genera.

Group 4 was also comprised of OTUs which were not present or rare in the minerals and roots prior to burial but which responded quickly (especially in the first timepoint) to the new environment. These were mostly affiliated with bacteria known for their copiotrophic lifestyle. The big increase in relative abundance early in the experiment was soon followed by a drop, remaining nevertheless the most important group in terms of relative abundance. Both compartments of the complex carbon

addition experiment shared more OTUs (17 OTUs) than minerals of both experiments (6 OTUs) (Figure 41). Nevertheless, in terms of contribution, minerals of both experiments were more similar. They shared 8 OTUs from the actinobacterial genera *Paenarthrobacter* and *Arthrobacter* that were the most important early stage colonizers of the minerals in both experiments, accounting for 48.4% to 26.4% of the bacteria present in minerals of the simple carbon addition experiment and 36.5% to 13.8% of the complex carbon addition experiment.

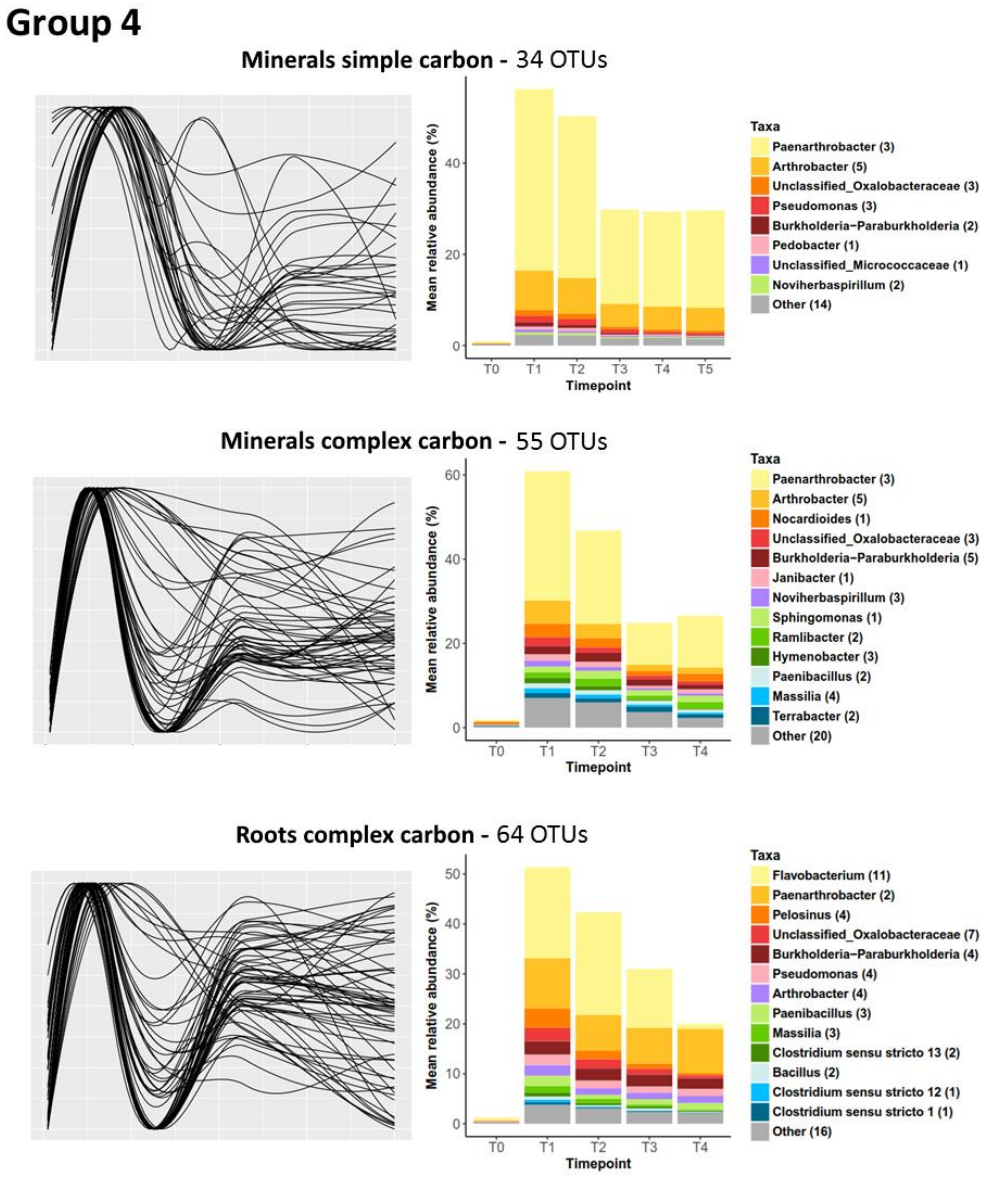

Figure 41 – OTUs which compose group 4 and their taxonomic affiliation at genus level, split according to experiment and compartment. Numbers in brackets represent how many OTUs represent the depicted genera.

Group 5 reflected OTUs which, once again, were mainly not present or rare in the mineral and root material prior to burial but had an increase in relative abundance until the middle of the experiments, where they peak. After this a decrease was registered. These OTUs are mainly distinct for the different sample types and experiments and seemed more important for minerals of the simple carbon addition experiment (relative abundance of 22.5% at timepoint 3), with *Terrabacter*, *Streptomyces* and *Kribella* being the most important genera (Figure 42).

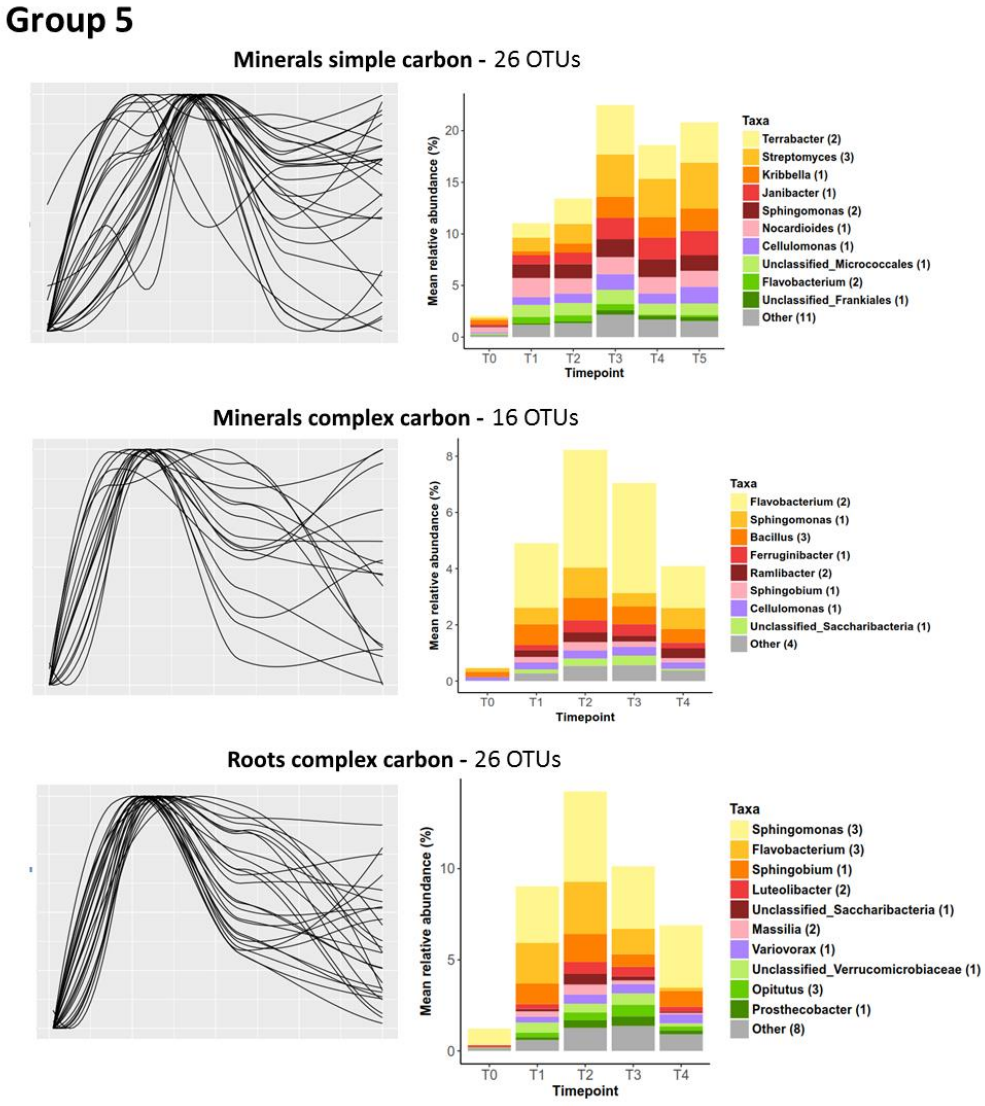

Figure 42 – OTUs which compose group 5 and their taxonomic affiliation at genus level, split according to experiment and compartment. Numbers in brackets represent how many OTUs represent the depicted genera.

The last group identified (group 6) is similar to both groups 4 and 5, but the peak in relative abundance occurs late in the experiments. These OTUs, as was the case for group 5, were distinct

between sample types and experiments (Figure 43). *Sphingomonas* and unclassified genera of *Sphingomonadaceae* contributed the most for minerals the simple carbon addition experiment, while *Flavobacterium* and *Streptomyces* were predominant in minerals of the complex carbon addition experiment. Regarding the roots, these were dominated by *Streptomyces*, *Herbaspirillum* and *Filimonas*.

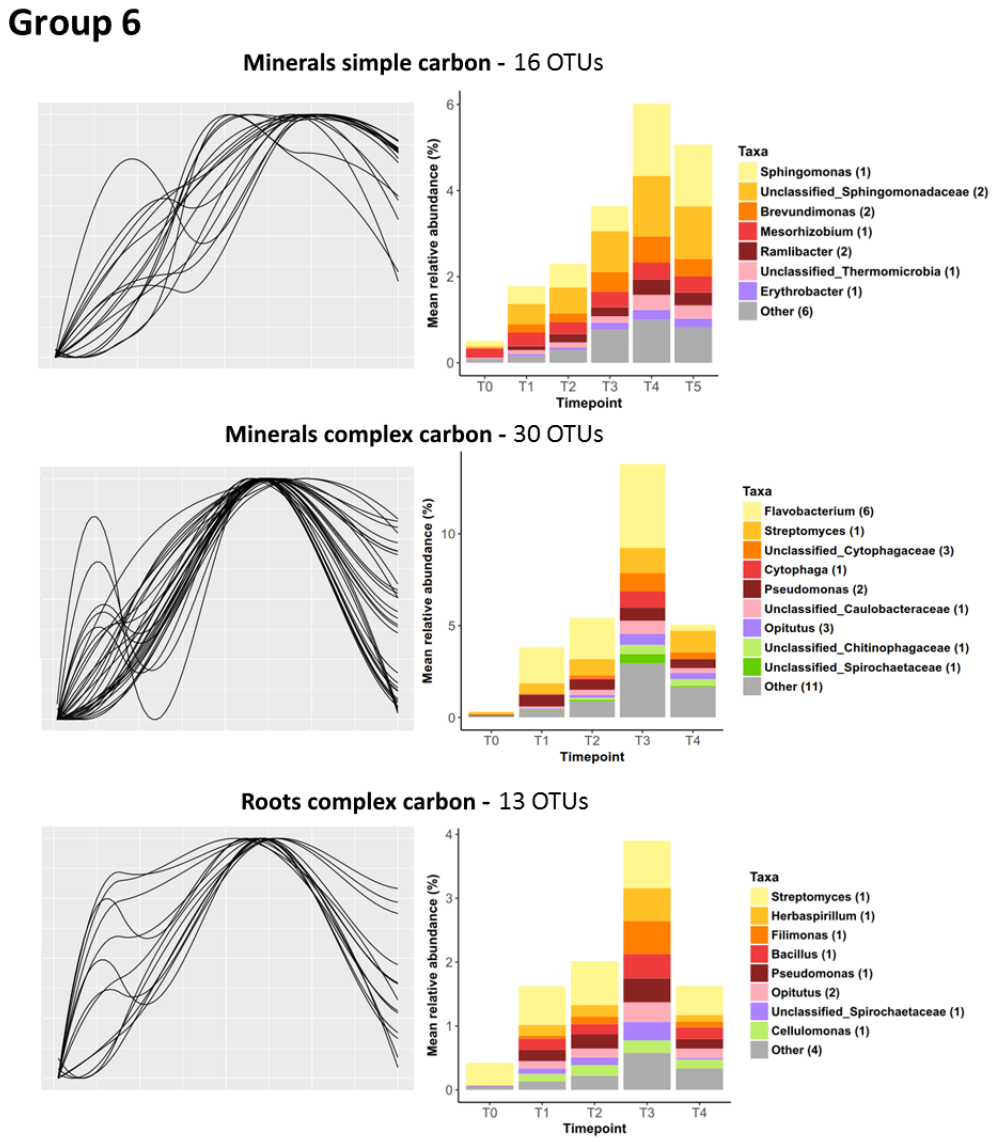

Figure 43 – OTUs which compose group 6 and their taxonomic affiliation at genus level, split according to experiment and compartment. Numbers in brackets represent how many OTUs represent the depicted genera.

5.1.4 – Some OTUs are distinct between minerals of both experiments

Although many OTUs were shared between minerals, some OTUs could be distinguished as to being present only in the simple carbon addition experiment (35 OTUs) or only in the complex carbon addition experiment (82 OTUs) (Figure 44). These made up the maximum of 6.6% of minerals of the simple carbon addition experiment at timepoint 4 and belonged to 28 different genera, with *Nocardioides*, *Lysobacter* and *Rhodococcus* being the most contributing. Regarding the minerals of the complex carbon addition experiment, these OTUs represented a bigger fraction of all OTUs, reaching a maximum of 18.9% at timepoint 3. The most abundant group of these OTUs belongs to the bacterial genus *Flavobacterium* and they are also found in the root material at even bigger relative abundances than in the minerals. Other abundant bacterial genera were *Opitutus* and *Paenibacillus* and members of the *Cytophagaceae* family.

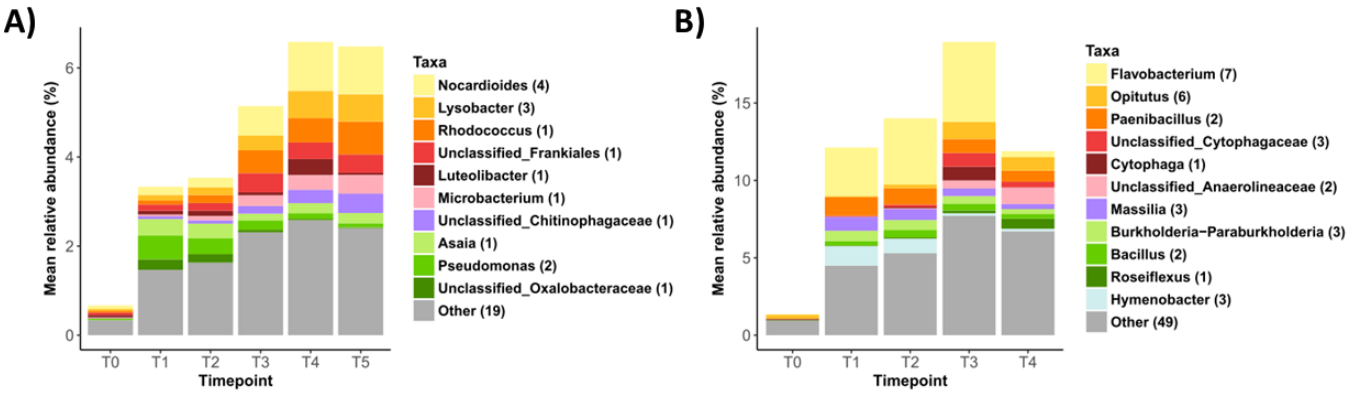

Figure 44 – Abundant OTUs which were unique to minerals of A) the simple carbon addition experiment or B) the complex carbon addition experiment. Numbers in brackets represent how many OTUs represent the depicted genera.

5.1.5 – Selection processes are stronger in mineral and roots than in surrounding soils

In order to investigate the relative influences of stochastic and deterministic (e.g. selection) processes governing the bacterial succession in control, adjacent soil, minerals and roots, the phylogenetic turnover between communities was determined calculating abundance-weighted β Mean Nearest Taxon Distances (βMNTD). Then a null modelling approach was employed which generates an expected level of βMNTD given a dominance of stochastic processes (random shuffling of species on the phylogenetic trees). For quantification of the magnitude and direction of deviation from the calculated βMNTD to the expected βMNTD, the β Nearest Taxon Index (βNTI) was calculated (Stegen *et al.* 2013).

For all sample types in both experiments, stochastic processes dominate the succession of bacterial communities in comparison to selective processes (Table 7, Table 8). Nevertheless, the proportion of selective impact on the temporal turnover of bacterial communities is consistently larger in the mineral and root compartments when compared to the control and adjacent compartments (Table 8, Table 9).

Table 8 - Influence of stochastic and deterministic assembly processes on the bacterial communities of the simple carbon addition experiment between timepoints. The governing processes were determined using βNTI estimated from relative abundances at genus level (Stegen *et al.* 2013). Percentages represent the relative amount of pairwise comparisons for which -2 < βNTI > 2 (stochasticity) or -2 > βNTI < 2 (selection).

			control		
	T0-T1	**T1-T2**	**T2-T3**	**T3-T4**	**T4-T5**
selection	3.3%	4.6%	3%	13%	15%
stochasticity	96.7%	95.6%	97%	87%	85%
			adjacent soil		
selection		14%	4%	6%	6%
stochasticity		86%	96%	94%	94%
			minerals		
selection	11.1%	20%	18.9%	16.7%	16%
stochasticity	88.9%	80%	91.1%	83.3%	84%

Table 9 - Influence of stochastic and deterministic assembly processes on the bacterial communities of the complex carbon addition experiment between timepoints. The governing processes were determined using βNTI estimated from relative abundances at genus level (Stegen *et al.* 2013). Percentages represent the relative amount of pairwise comparisons for which -2 < βNTI > 2 (stochasticity) or -2 > βNTI < 2 (selection).

	control			
	T0-T1	**T1-T2**	**T2-T3**	**T3-T4**
selection	6%	5%	4%	15%
stochasticity	94%	95%	96%	85%
	adjacent soil			
selection		5%	7%	8%
stochasticity		95%	93%	92%
	minerals			
selection	10%	30%	31%	31%
stochasticity	90%	70%	69%	69%
	roots			
selection	20%	11%	14%	16%
stochasticity	80%	89%	86%	84%

5.2 – Discussion

5.2.1 – Mineral properties determine bacterial community structure

The capacity of distinct bacteria to specific carbon compounds can contribute for their successful adaptation to specific soil habitats. Bacteria have distinct metabolic capabilities and different bacterial communities assemble depending on substrate complexity (Kramer & Gleixner 2008; Koranda *et al.* 2014; Bonanomi *et al.* 2017). The response of soil bacteria to the addition of different carbon sources of varying chemical recalcitrance has been studied, and many more bacterial taxa were enriched by the addition of a labile substrate (glycine and sucrose) as compared to the addition of more recalcitrant substrates (cellulose, lignin and tannin-protein) (Goldfarb *et al.* 2011). This effect is verified in the present study, with bacterial communities in minerals of both simple and complex carbon addition experiments being distinct. Nevertheless, bacterial communities on the minerals were more similar to each other than to those in control and adjacent soils and roots. Therefore, mineral properties are a stronger driver than growth substrate complexity. This stronger effect has not before been described, even though differential colonization of distinct specific soil minerals by bacterial communities has been reported for microcosm experiments (Hutchens *et al.* 2010; Ditterich *et al.* 2016; Colin *et al.* 2017). The selective nature of minerals is due to their surface charge (Roberts 2004), roughness (Qiaoyun Huang *et al.* 2015) and chemical composition (Gleeson *et al.* 2006). This effect has been shown for the clay minerals illite in a study using different artificial mineral mixtures, where mixtures containing this mineral contained similar PLFAs and were distinct from others. The difference of illite to other clay minerals such as montmorillonite may stem from its lower cation exchange capacity and lower amounts of Ca, Mg, Na and K (Ditterich *et al.* 2016). For the iron oxyhydroxide goethite, most of the research has focused on its interaction with soil dissolved organic carbon and phosphate. Some work has traced the activity and biofilm development of specific bacteria, such as *Bacillus subtilis* (Ma *et al.* 2017), *Pseudomonas putida* (Wu *et al.* 2014) and *Shewanella oneidensis* (Yan *et al.* 2016) on this mineral, which generally enables the establishment of a smaller quantity and less active cells, when compared to other minerals. Only one report has evaluated the microbial colonization of goethite, associating it with distinct bacterial and fungal communities when compared with quartz controls (Heckman *et al.* 2013). Taken together with the results of the present study, this information shows that minerals have a role in microbial ecology that surpasses their function of simply providing an inert matrix for microbial growth.

Despite the similarities observed between mineral bacterial communities, there are several bacterial OTUs which preferentially colonize the minerals in the presence of complex carbon but not when simple carbon compounds are present. These OTUs likely represent bacteria responsible for the

specific degradation of plant root material and belong mainly to the *Flavobacterium* genus and the *Cytophagaceae* family. These bacterial taxa are ubiquitous in soils and are frequently reported as degraders of several polysaccharides, including cellulose and hemicelluloses, the main components of dead plant material (Štursová *et al.* 2012; Chen *et al.* 2013; Kramer *et al.* 2016; Bai *et al.* 2017). In a recent study, members of both of these bacterial taxa were found to be dominant in semi-desert soils after amendment with crystalline cellulose (Kumar & Khanna 2014). Apart from known plant degraders, other taxa such as *Opitutus* were also found exclusively associated with the minerals to which complex carbon substrates had been added. Although soil bacteria from the *Verrucomicrobia* phylum are relatively understudied, some members, especially from the family *Opitututaceae* have been reported to have the capacity of degrading polysaccharides (Chin *et al.* 2001; Talamantes *et al.* 2016) and to be active in the degradation of plant material (Pepe-Ranney *et al.* 2016; Wilhelm *et al.* 2017).

5.2.2 – Bacterial colonization of newly introduced minerals in soil is a deterministic process

The mechanisms which mediate the colonization of minerals by soil microorganisms are important for a better understanding of the process of soil formation. The results of the present study can be viewed in the light of metacommunity theory, a framework which attempts to explain the interdependence of local interactions and regional processes (e.g. dispersal), by making use of four conceptual models: neutral model, patch dynamics, species sorting and mass effect (Logue *et al.* 2011). Obviously, the neutral model can be excluded based on the data obtained in the current study. Despite some potential non-homogeneous distribution of roots and minerals in the complex carbon addition experiment, the overall homogeneity of the introduced corer material appears to be high enough to minimize a patch dynamic effect within the corers on their overall communities. Similarly, and although dispersal is likely high, it is not enough to create mass effects (high dispersal rates ensure a constant supply of new colonizers to sites normally considered marginal or outside of their environmental range), since there is a temporal dynamic within mineral and root communities which does not occur in the surrounding soils. Species-sorting occurs if resource gradients or habitat cause sufficiently strong differences in local demography and species interactions (Leibold *et al.* 2004) through competitive exclusion (Guichard 2017) and dispersal rates are high enough to enable species to rapidly colonize niches within the novel habitat (Winegardner *et al.* 2012). This can explain the current results best, since dispersal is obviously occurring at high rates, with the mineral and root communities prior to burial being almost completely substituted by organisms which did not exist in these materials before and minerals seem to provide a selective environment, enabling

the establishment of adapted bacterial communities (Figure 32, Tables 8 and 9). Moreover, the most abundant OTUs inside the containers are not necessarily abundant in the surrounding soils, emphasizing selective effects.

Besides the selection by minerals, one cannot exclude the role of dispersal limitation. The minerals are inserted inside containers which can provide a barrier to the movement of water and of soil fauna and stochastic processes were found to have a big influence in the mineral communities. It is therefore likely that dispersal limitation is a factor at play in these environments, as was the case in a recent study which investigated the colonization of bags filled with minerals (ferrihydrate, kaolinite and quartz), inserted in pots where plants of *Avena barbata* where grown. Analysis of bacterial, fungal and archaeal communities growing in distinct mineral fractions revealed that dispersal limitation was an important factor structuring the microbial communities in all minerals investigated (Whitman *et al.* 2017).

The active bacterial communities on the minerals suffer a big turnover upon insertion in the soil environment, with the most abundant OTUs being rapidly replaced by others in both experiments. This shows a lack of adaptability of these OTUs to the shifting environment, even though they were present in the minerals in high numbers, suggesting a small role of priority effects. Although not well explored for soil bacteria, priority effects were shown to affect microbial community richness and increase the local prevalence of taxa with high dispersal capacities or high abundances (Symons & Arnott 2014; Werner & Kiers 2015; Sprockett *et al.* 2018). The findings of this study are in contrast with a recent investigation regarding the sources of soil microbial colonization of a receding alpine glacier. Here the establishment of aerosolized microbes on the receding glacier soil was limited by glacier-related microorganisms which had already occupied their niches, indicating a role of priority effects in determining initial microbial communities trajectories during soil formation (Rime *et al.* 2016).

The findings of this study provide novel insights into the colonization of newly available soil environments, but these studies need to be extended to other soils and mineral types with distinct properties.

5.2.3 – Abundant mineral bacterial colonizers display distinct temporal activity patterns irrespectively of taxonomic affiliation

In the present study, it was possible to distinguish the activity patterns of 461 abundant bacterial OTUs which have a role on the colonization of novel mineral surfaces in soil.

The most important OTUs in both experiments belong to group 4 and represent the bacteria that respond quickly to the presence of the minerals (first colonizers). These bacteria are likely sustained in the minerals by the nutrients added and by the decaying biomass the bacteria present in the minerals prior to burial (group 1) which could not adapt to the novel conditions. In fresh mineral surfaces in soils, the first colonizers are reported to be fast-growing bacteria which outcompete the more fastidious ones (Ditterich *et al.* 2016; Pronk *et al.* 2017). This is in agreement with the present study and is particularly observed for the copiotrophic *Paenarthrobacter* and *Arthrobacter*. The majority of the cultured isolates from these taxa are able to grow at high nutrient conditions (*Paenarthrobacter* was only very recently reclassified as an independent genus, being previously associated to *Arthrobacter* genus; Busse, 2016)). Although these bacteria suffer a decrease at later stages, *Paenarthrobacter* and *Arthrobacter* remained abundant in the minerals throughout the experiments, even in the simple carbon addition experiment where the carbon sources added are expected to be rapidly metabolized (Kramer *et al.* 2016). This maintenance of high activity is likely related to the specific ability of *Arthrobacter* species to weather minerals in order to release cationic key nutrients from them. This ability has been verified for this group with calcite (Colin *et al.* 2017), granite(Frey *et al.* 2010), potassium feldspar and biotite (Lepleux *et al.* 2012; Zhi Huang *et al.* 2015), and it is possibly key in providing vital nutrients to sustain the remaining members of the mineral bacterial communities. After the depletion of easily accessible carbon sources, it is likely that oligotrophic bacteria have colonization advantage and become abundant. These bacteria are adapted to low-nutrient environments and probably obtain their nutritional requirements from leachates from the upper soil layer, the cations weathered by the *Arthrobacter* species and from biomass of meanwhile dead first colonizers. This is reflected on bacteria from groups 3 and 6, which have a slow development in initial stages but peak later in the experiments. Many of the constituents of these groups are reported to be fastidious, such as *Chloroflexi* (Davis *et al.* 2011) and *Verrucomicrobia* (Bergmann *et al.* 2011).

Despite the existence of a big array of studies of the bacterial diversity in soils worldwide, it is still very difficult to link this information to a functional role and therefore make ecological interpretations. Some bacterial groups like *Rhizobium* (Denison & Kiers 2011; Gourion *et al.* 2015) and *Azospirillum* (Bashan & De-Bashan 2010; Fibach-Paldi *et al.* 2012) are well studied, but they make up a small fraction of all bacteria and mostly represent functions beneficial or detrimental to plant function. Over 10 years ago, researchers have attempted to divide soil bacterial phyla into categories based on *r*- to *K*-selection continuum (Fierer *et al.* 2007). Although useful, it is a very course classification which does not represent all individual members of each group, since each phylum comprises an enormous level of phylogenetic and physiological diversity. The authors

acknowledge this fact, even identifying some phyla for which these categories may not apply, such as for *Alphaproteobacteria*, *Firmicutes* and *Actinobacteria* (Fierer *et al.* 2007). This is substantiated by the present study for bacteria from the same genera, such as *Cellulomonas*, *Nocardioides*, *Ramlibacter* and *Sphingomonas,* which display distinct activity patterns, with some OTUs corresponding to groups 4 and 5 which represent fast responding bacteria, and some others matching to group 3 which represent bacteria slowly growing throughout the experiments. In a similar manner, some OTUs which are affiliated to genera not usually reported as oligotrophs, such as *Nocardioides* and *Flavobacterium* were found in groups 3 and 6. It is evident that closely related bacteria can have distinct behaviour under the same conditions and therefore investigations at higher taxonomic resolution are necessary for the prediction of early colonization of mineral surfaces.

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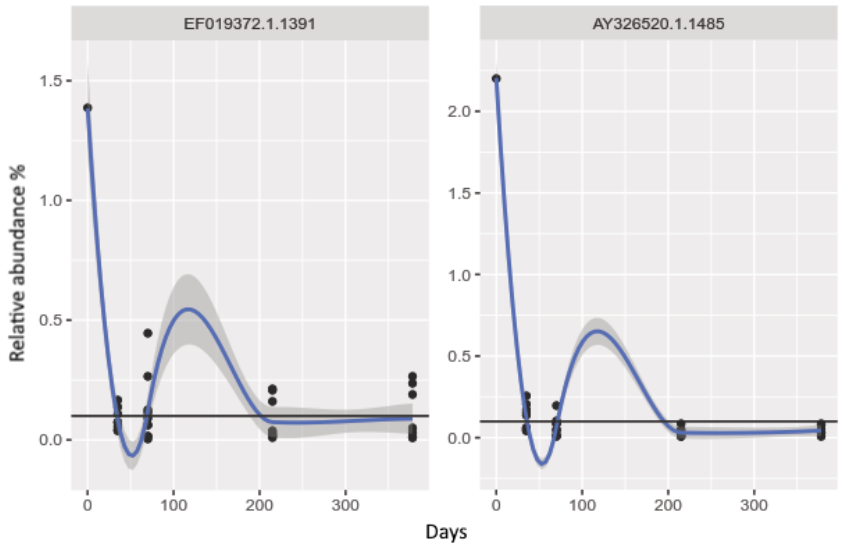
5.4 – Supplementary information

Supplementary Figure 1 – Example of two OTUs from the roots of the complex carbon addition experiment for which an artificial increase in relative abundance after the initial decrease was register with LOESS curve fitting (blue line). Points represent the relative abundance of the OTU in the different plots throughout time. Black line marks 0.1% relative abundance.



Chapter 6 – Cultivation of novel soil bacteria

6.1 – Results

Cultivation of novel bacteria is typically a matter of random chance. The parameters which might enable a more efficient isolation of novel bacteria are rarely systematically explored. This chapter attempts to investigate the effect of different parameters on the potential to isolate novel bacteria, with a focus on oligotrophic soil bacteria. To do so, highly diluted soil suspensions from 6 different soils were subjected to enrichment and isolation approaches using three different methods targeting different bacterial lifestyles in combination with up to four different, well defined low nutrient media. The methods comprise enrichments via high-throughput liquid culturing and a biofilm formation approach on different surfaces such as polypropylene, polystyrene, steel and glass. From both enrichments isolates were obtained. The third method comprised direct plating of highly diluted cellular suspensions. The data were evaluated with respect to efficiency of methods in obtaining isolates, specifically novel isolates. Further experimental details are given in Experimental Procedures (Chapter 3).

6.1.1 – Bacterial community structure in selected soil samples

Six soil samples were selected for cultivation, representing the diverging conditions encountered in the Biodiversity Exploratories locations, in terms of soil characteristics and land use regimes.

The selected samples were analysed using next-generation amplicon sequencing of the V3 region of the 16S rRNA gene to determine the structure of the bacterial community per sample. After quality filtering, denoising and chimera removal, approximately 5,400,000 sequences were obtained which were clustered at 99% sequence similarity using QIIME open reference approach against the SILVA SSU Ref 128 database at 99% sequence identity. Sequences assigned to chloroplast were removed and the remainder could be clustered in 84,371 operational taxonomic units (OTUs). Near saturation was observed for the rarefaction curves performed for the OTUs present in the individual samples, (Figure 45A). An exception was sample AEW08 which did not reach saturation. Sample coverage estimates were between 96.7% – 99.3% for the six soils (median value 98.8%), which indicated that our sequence inventory covered most of the OTUs present in the samples. When looking at rarefaction curves at other taxonomic levels, the totality of the taxa were covered (Figure 45B). After calculation of rarefaction and alpha diversity metrics, counts were normalized.

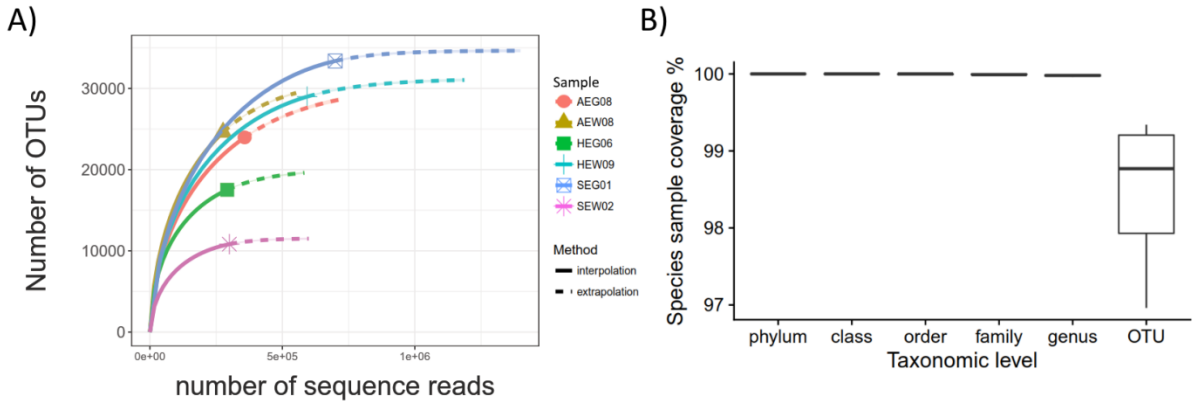


Figure 45 – A) Rarefaction curves for the six different samples chosen for cultivation at OTU level. B) Species sample coverage estimates for all taxonomic levels.

The bacterial communities present in the different samples were analysed with respect to alpha diversity (Figure 46). The samples chosen were distinct regarding the number of distinct OTUs. Samples SEW02 and HEG06 had the lowest value of richness, harbouring approximately three or two times less OTUs, respectively, than the highest value observed, for the sample SEG01.

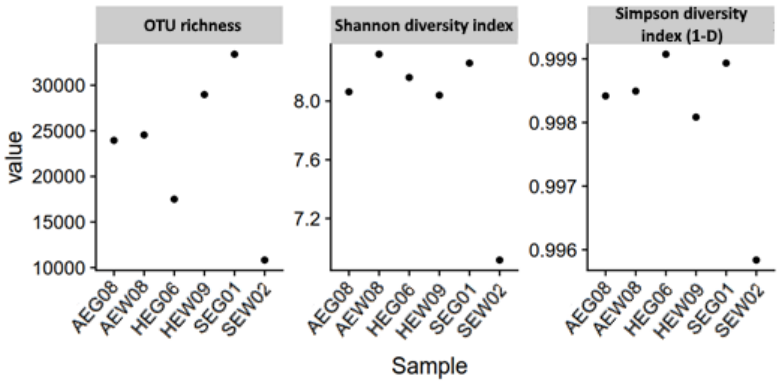


Figure 46 – Alpha diversity measures for bacterial communities at OTU level, for the six different soil samples chosen for cultivation.

The determinants of bacterial community composition were evaluated using NMDS based on weighted UniFrac distances at the OTU level (Figure 47A). Bacterial communities on the different samples were distinct but cluster according to their origin (forest versus grassland soil). This was also reflected in terms of relative abundance of bacterial phyla and proteobacterial classes (Figure 47B). In general samples which originate from forest soils have a higher proportion of *Acidobacteria*, *Alphaproteobacteria* and *Gammaproteobacteria* and a lower number of *Actinobacteria*, *Bacteroidetes* and *Deltaproteobacteria*.

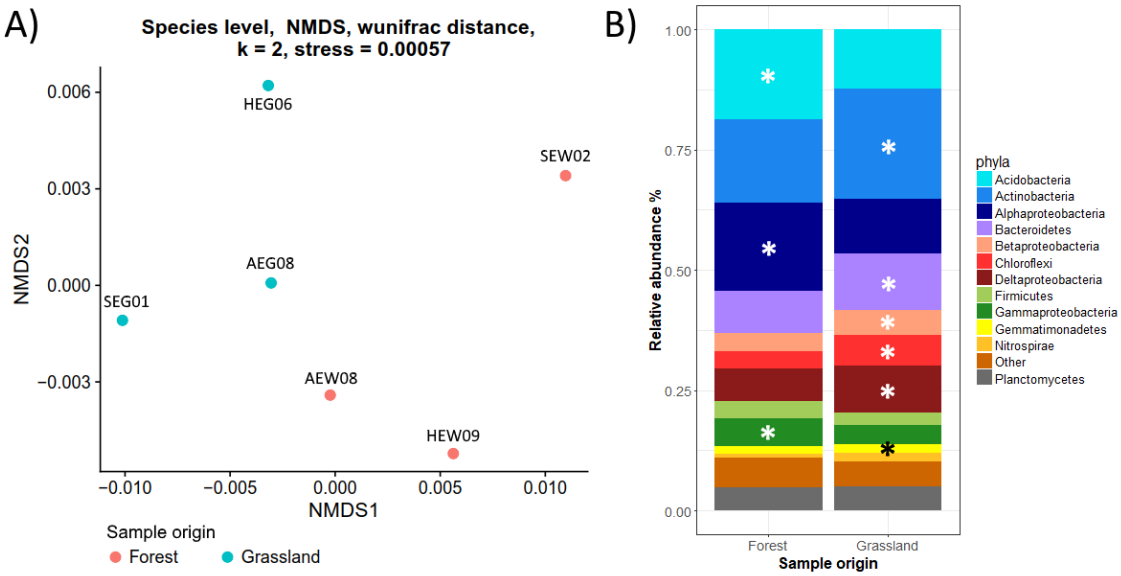


Figure 47 – Bacterial community structure and diversity on the soil samples selected for cultivation. A) NMDS plot of bacterial composition based on weighted UniFrac distances at OTU level, coloured by sample origin. B) Average relative abundances of bacterial phyla and proteobacterial classes in forest and grassland soil samples. Taxa with significant differential distribution (detected with t test ($p<0.01$) are marked with asterisks.

6.1.2 – High-throughput dilution in liquid media

Liquid media dilution was employed in a high throughput fashion in order to target oligotrophic bacteria that readily grow in liquid conditions. Inoculation of a low number of cells (N=2, N=5) was employed as a means to decrease competition by fast-growing bacteria. Four different media compositions were used to account for the different nutritional needs of distinct bacteria. The highest number of grown wells was observed for SSE/Cmix medium while the lowest was detected on SSE/Polymermix (Table 10).

Table 10 – Inoculated and grown wells from the different media with the high throughput liquid media dilution cultivation approach.

Medium	Nº inoculated microtiterplates	Nº inoculated wells	Nº grown wells	Average % of grown wells per microtiterplate
BEX/HD1:10	12	720	160	23.2
SSE/Cmix	12	720	208	31.9
SSE/HD1:10	12	720	131	18.6
SSE/Polymermix	12	720	112	15.4

Culturability of aerobes in the different media was plotted (Figure 48), except in the cases where fungal contamination was observed in the plates. There was no evident relationship between the number of cells inoculated and the culturability values, as for BEX/HD1:10 and SSE/Polymermix culturability was higher when 2 cells were inoculated. The opposite was verified for SSE/Cmix and SSE/HD:10. Highest culturability (32.2%) was obtained for sample AEG08 in SSE/Cmix medium when inoculated with 5 cells per well. A multiple linear model was applied in order to identify the parameters that drive culturability and it was confirmed that SSE/Cmix has a positive effect on culturability ($p < 0.01$). Also, the samples AEG08 (Leptosol) and HEG06 (Stagnosol) were positively associated with culturability ($p < 0.01$).

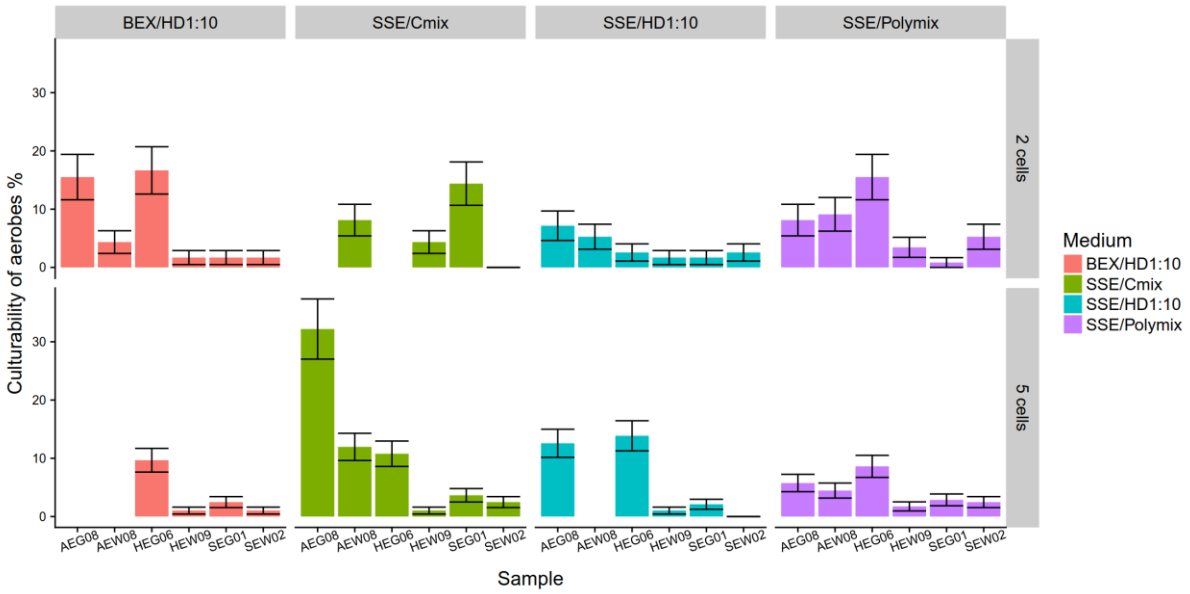


Figure 48 – Percentage of culturability of aerobes in high throughput liquid dilution cultivation, by medium and number of cells inoculated per well, based on most probably number (MPN). Whiskers represent standard deviation. Missing bars represent experiments for which a fungal contamination was observed in some wells.

After incubation the bacterial community present in the wells showing growth was analysed by a barcoded Illumina paired-end sequencing method targeting the 16S ribosomal RNA V1-2 hypervariable region. After quality filtering, denoising and chimera removal, approximately 5,750,000 sequences were obtained which were clustered at 99% sequence similarity using QIIME open reference approach against the SILVA SSU Ref 128 database at 99% sequence identity. Sequences assigned to chloroplast were removed and the remainder could be clustered in 12421 operational taxonomic units (OTUs). Sample coverage estimates were between 85.9% - 99.9% (Figure 49), but the vast majority was above 99%, which indicated that our sequence inventory

covered most of the OTUs present in the wells. The sample coverage estimates also indicated that the missed OTU were represented by a low amount of cells, hence are basically neglectable.

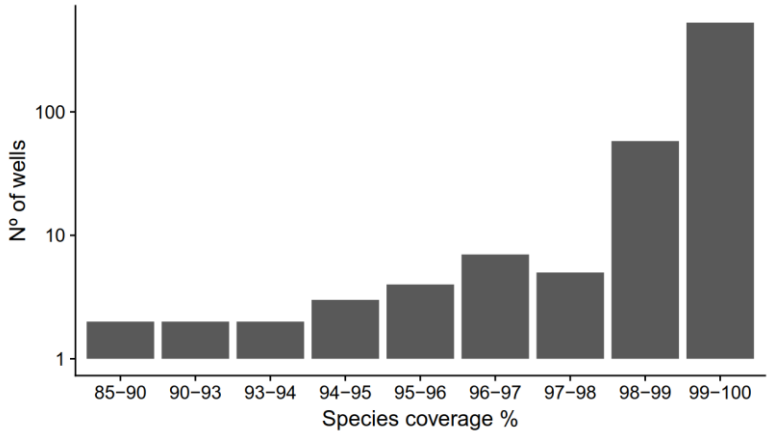


Figure 49 – Species sample coverage of the OTUs growing in each well of the high throughput liquid medium dilution approach (nº of wells is in logarithmic scale).

Near saturation was observed for the rarefaction curves performed for the OTUs present in the different media, which again indicated that our sequence inventory covered most of the taxa present in the samples (Figure 50). Species sample coverage was 99.9% for all media.

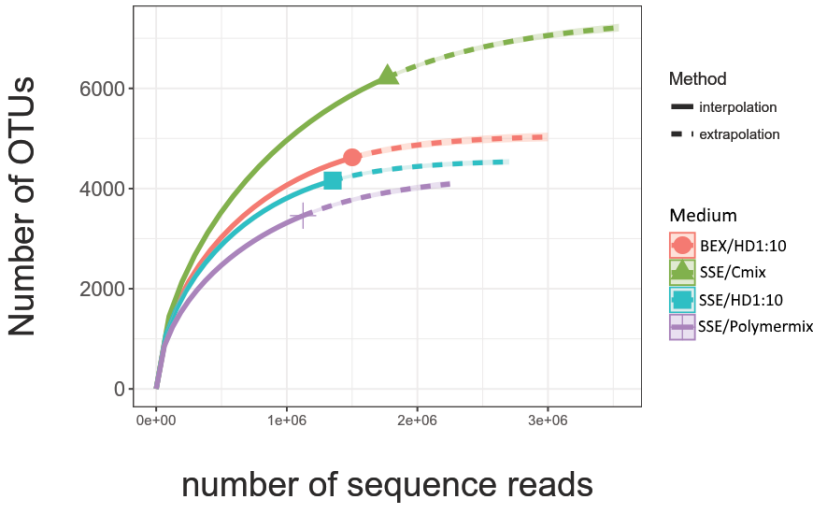


Figure 50 – Rarefaction curves for the OTUs growing in the four different media.

The relative abundance of each bacterial OTU growing in each individual well was calculated and all the OTUs with less than 1% in relative abundance were eliminated from analysis, since these should represent bacteria that could not grow in the provided media. After this filtering, alpha diversity metrics were calculated (Figure 51). The SSE/Cmix and SSE/Polymermix media harboured richer and more diverse communities when compared to the two media BEX/HD1:10 and SSE/HD1:10.

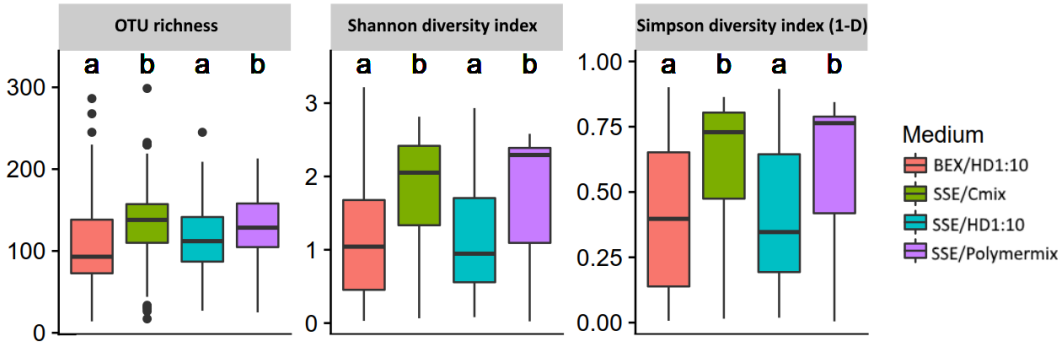


Figure 51 – Alpha diversity measures for bacterial communities at OTU level, for the 4 different media used in the high-throughput liquid media dilution cultivation. Letters denote significance (p< 0.05) as a result of multcomp test.

This was also the case when looking at the distribution of bacterial OTUs growing in the wells from each medium (Figure 52). Less bacterial OTUs grew in BEX/HD1:10 (mean of 5 OTUs/well) and SSE/HD1:10 (mean of 4.7 OTUs/well) when compared with SSE/Cmix (mean of 8.3 OTUs/well) and SSE/Polymermix (mean of 8.4 OTUs/well). Interestingly the distribution of OTUs in the both media containing HD1:10 is unimodal right-skewed, while distinct from the bimodal gaussian distributions observed for SSE/Cmix and SSE/Polymermix.

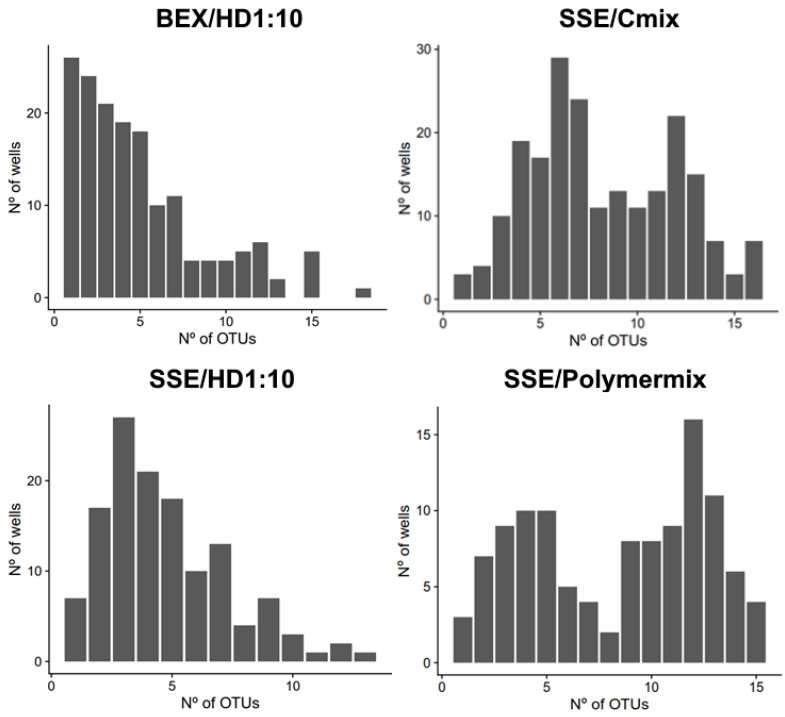


Figure 52 – Alpha diversity measures for bacterial communities at OTU level, for the 4 different media used in the high-throughput liquid media dilution cultivation.

Bacterial community composition was investigated for the SSE/Cmix and SSE/Polymermix using NMDS based on weighted UniFrac distances at the OTU level, in order to determine if the binomial distribution of OTUs observed in Figure 52 was associated with beta diversity (Figure 53). Communities were distinct for the different groupings, although for SSE/Polymermix they were not significant when employing a PERMANOVA test. This suggests that the different bimodal peaks observed in the SSE/Cmix and SSE/Polymermix histograms of Figure 52 might be characterized by different community structures.

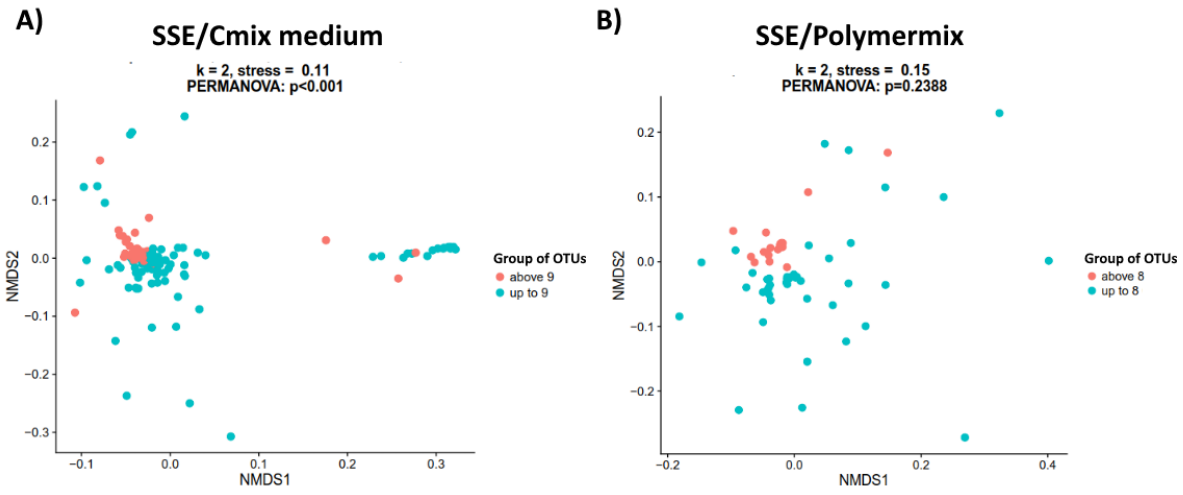


Figure 53 – NMDS plot of bacterial composition based on weighted UniFrac distances at OTU level for A) SSE/Cmix (PERMANOVA $p < 0.001$) or B) SSE/Polymermix (PERMANOVA $p = 0.2388$) medium. Colours reflect the different OTU groupings, based on the histograms of Figure 8.

Bacterial community composition was investigated using NMDS based on weighted UniFrac distances at the OTU level (Figure 54A). Bacterial communities cluster according to media (PERMANOVA $p<0.001$), but it is not clear if communities growing in BEX/HD1:10 and SSE/HD1:10 are more similar to each other than to the ones growing in other media. But when looking at the taxonomy of these OTUs, at order level (Figure 54B), some similarities can be found, namely a higher proportion of *Oceanospirillales* and *Bacillales* growing on SSE/Cmix (average of 29.3% and 11.7%, respectively) and SSE/Polymermix (average of 35% and 17.9%, respectively) when compared to the remaining media (average of 12.2% and 2.9% in BEX/HD1:10, and 10.2% and 2.7% in SSE/DH1:10, respectively). SSE/Polymermix medium seemed to sustain different bacteria than the other media. *Flavobacteriales* and *Burkholderiales* especially, were hardly present in this media (average of 0.9% and 0.9% versus 9.7% – 24.3% and 4% – 12.6%, respectively), and *Xanthomonadales* numbers were

significantly decreased (average of 3.5% versus 7.5% – 12.5%). On the other hand, *Micrococcales* were particularly enriched (average of 13.9% versus 4.2% – 7.6%).

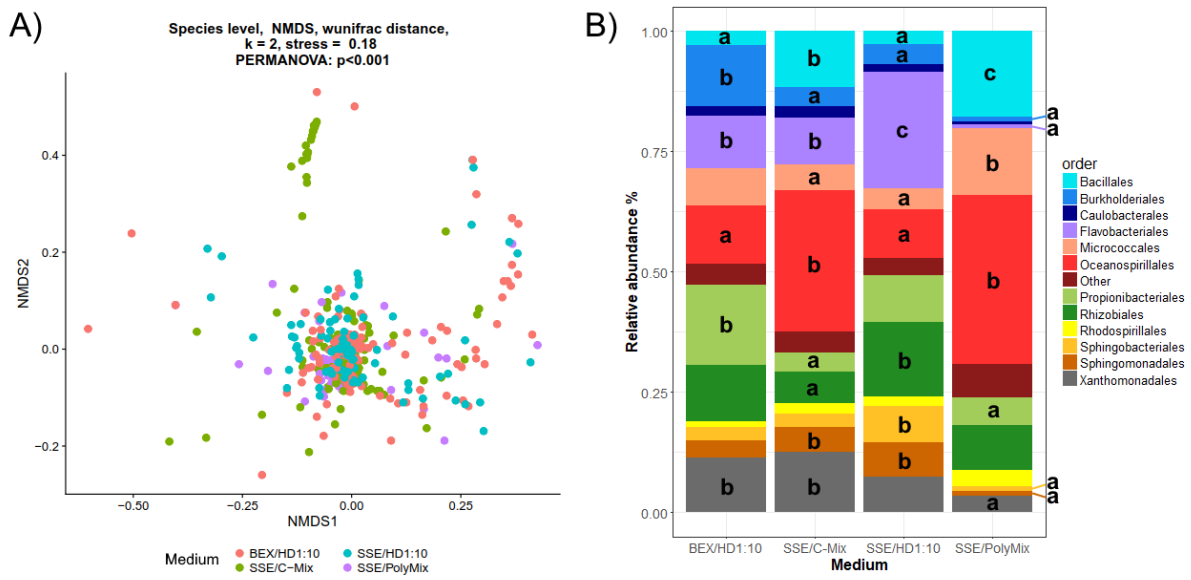


Figure 54 – Community structure and diversity growing on the wells in the different media. A) NMDS plot of bacterial composition based on weighted UniFrac distances at OTU level, coloured by medium. B) Average relative abundances of bacterial orders in the four different media. Letters denote significant differences as a result of the multcomp test.

The use of high throughput cultivation allows the systematic analysis of the specific relationships between the co-cocultures growing in the wells. This may reveal essential interactions for growth in the media used and which could be exploited for future cultivation attempts.

In order to evaluate if different genera growing in the wells occur more frequently than what would be expected, correlations were determined for all pairs of genera and compared to null models. 145 genera were investigated, and significant positive and negative relationships were determined for 43 of them. 210 significant positive relations were detected (Figure 55). The bacterial genera which were found to co-occurred with the biggest number of other genera were *Aeribacillus*, *Oceanobacillus*, *Nesteronkia* and *Caldalkalibacillus* all positively associated with 10 other genera.

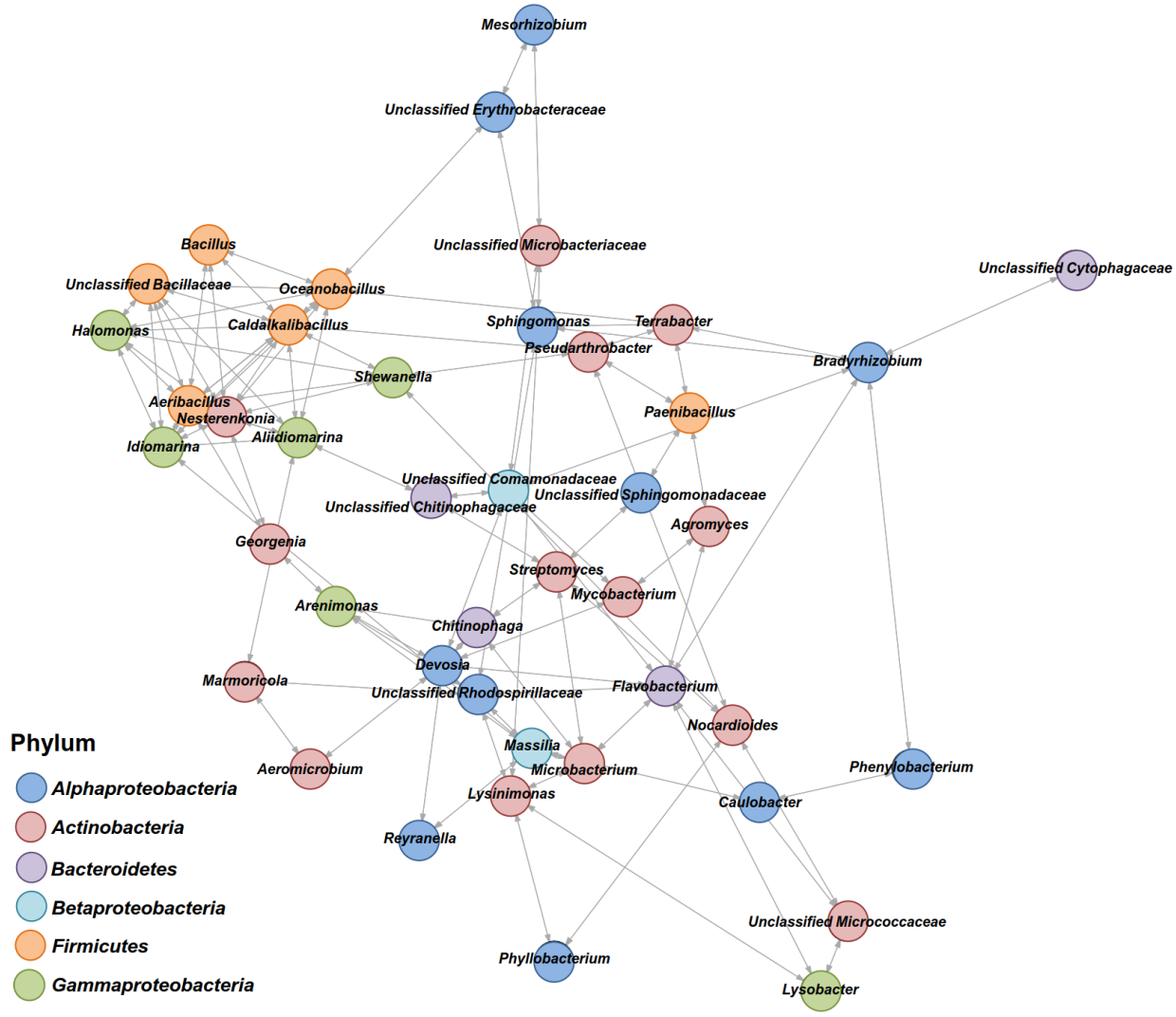


Figure 55 – Network depicting significant ($p < 0.01$) positive associations between bacterial genera growing in the wells of the high throughput liquid media dilution cultivation. Colours denote the bacterial phyla to whom the genera belong.

Many more significant negative correlations were detected (832) when compared to the positive ones (Figure 56). *Georgenia* was the main player here, establishing 38 putative negative interactions with other genera. *Bacillus* and unclassified members of *Micrococcaceae*, *Cytophagaceae* and *Erythrobactereaceae* were also important potential antagonists of bacterial growth, with 32, 35, 35 and 31 negative correlations, respectively, being detected.

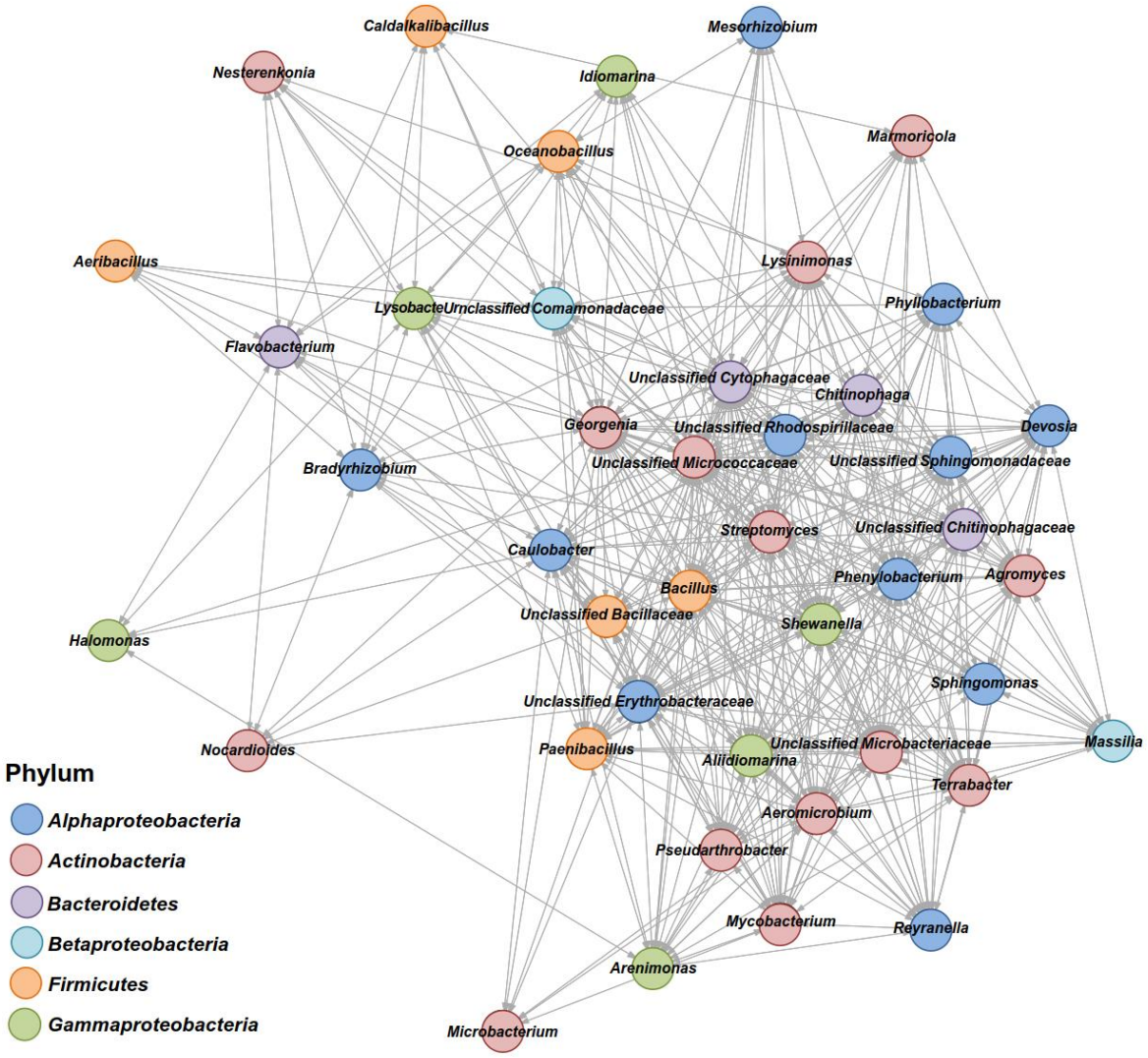


Figure 56 – Network depicting significant ($p < 0.01$) negative associations between bacterial genera growing in the wells of the high throughput liquid media dilution cultivation. Colours denote the bacterial phyla to whom the genera belong.

After assessment of the growing bacteria, several wells were chosen as target based on taxonomic novelty (Table 11). These wells had the most potential for allowing the isolation of novel oligotrophic bacteria. The medium with the largest fraction of chosen wells was SSE/HD1:10, with 17.6% of the wells being plated. On the other hand, SSE/Polymermix had the lowest number with only 8.9% of the grown wells being selected. After plating and incubation, partial sequences of the 16S rRNA gene were obtained for the colonies growing on the plates. After dereplicating the obtained sequences,

proportional distance was calculated based on pairwise deletion and the sequences were grouped. 97% sequence similarity was used as threshold to determine sequences belonging to different bacterial species. A total of 78 distinct species were obtained (Table 11), being that SSE/Cmix harboured the most diversity, with 31 different bacterial species being isolates. SSE/HD1:10 and BEX/HD1:10 allowed the isolation of 28 and 25 different species, respectively. Isolation did not reflect the richness and diversity estimated for the wells (Figure 51) as SSE/Polymermix was one of the media harbouring the richest and more diverse communities, but it yielded the least amount of species and isolates only allowing the isolation of 7 different species.

Table 11 – Chosen wells and isolated bacteria from the different media with the high throughput liquid media dilution cultivation approach.

Medium	Nº of chosen wells	Nº of isolated colonies	Nº of species	Nº of novel isolates < 97% 16S similarity	Nº of novel isolates < 95% 16S similarity	Total nº of new isolates
BEX/HD1:10	16	110	25	2	0	2
SSE/C-mix	33	134	31	5	5	10
SSE/HD1:10	23	140	28	4	1	5
SSE/Polymermix	10	22	7	0	0	0

The majority of species was specific to one medium (Figure 57), with only 16.7% being shared by different media. The highest number of isolates corresponding to the same bacterial species isolated from two different media were obtained from SSE/HD1:10 and BEX/HD1:10 (total of 8).

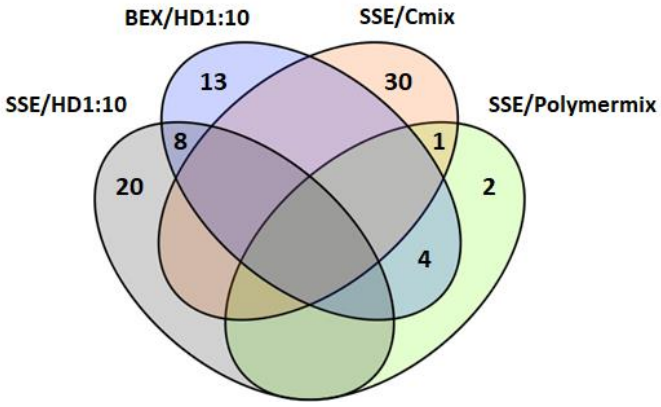


Figure 57 – Venn diagram depicting the shared bacterial species isolated from the 4 different media used in the high throughput liquid media dilution cultivation.

Rarefaction was plotted for the distinct species isolated from the different media (Figure 58). Sample coverage estimates were of 79% for SSE/HD1:10, 70.9% for BEX/HD1:10, 34.4% for SSE/Cmix and 5.3% for SSE/Polymermix. This showed that for the media supplemented with HD1:10, the isolation retrieved the majority of species which would be possible to cultivate under these conditions. Nevertheless, for SSE/Cmix and especially for SSE/Polymermix the vast majority of cultivable species could not be retrieved and therefore, further cultivation efforts with these media would yield many more species.

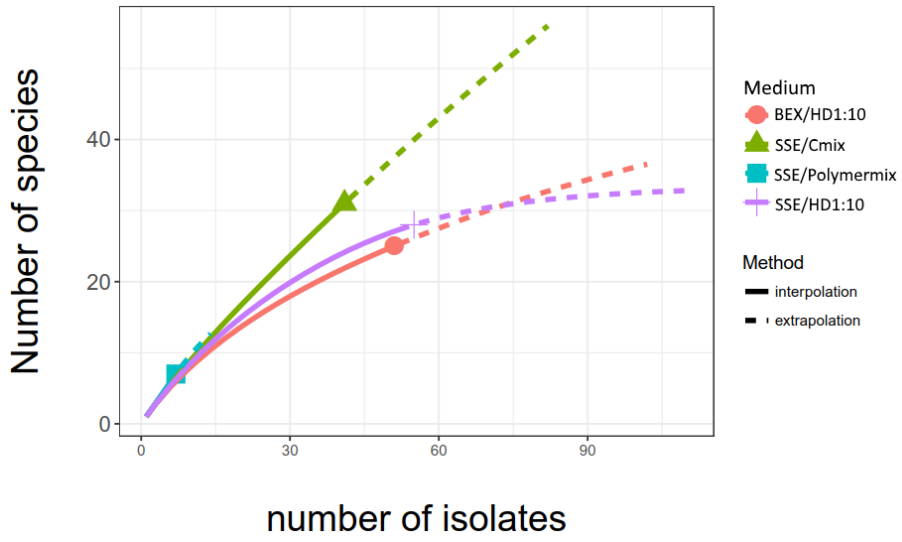


Figure 58 – Rarefaction curves for the isolated bacterial species from the four different media of the high throughput liquid medium dilution approach.

A co-occurrence analysis was also employed to the isolated species, in order to assess if specific pairs of bacteria were systematically isolated together (Figure 59). Some positive relationships were present, mostly between *Actinobacteria* and *Bacteroidetes* members. Interestingly, the association between *Flavobacterium* and *Agromyces* was also previously predicted for the enrichments growing in the wells, prior to isolation (Figure 55). None of the species with positive interactions were detected in the SSE/Cmix medium.

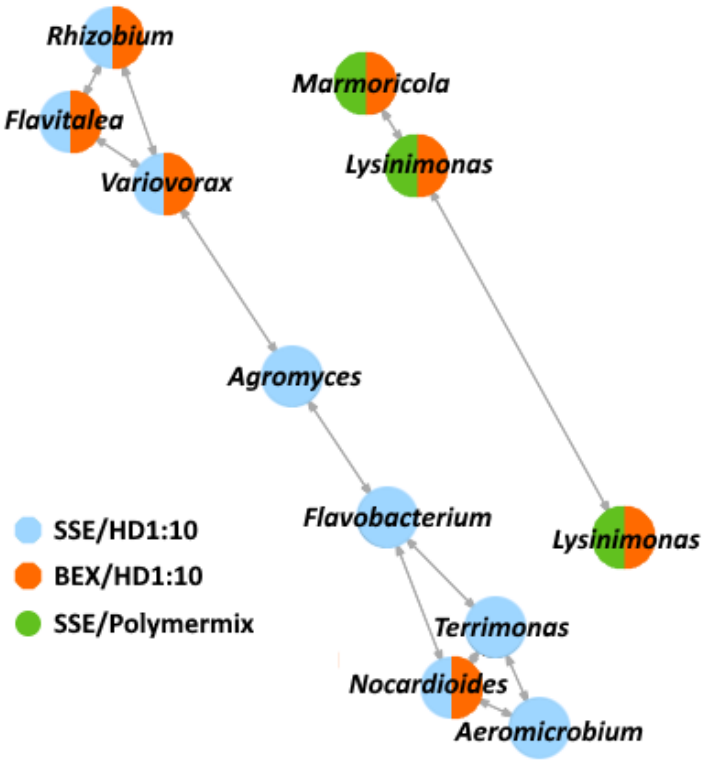


Figure 59 – Network depicting significant ($p < 0.01$) positive associations between bacterial species isolated from the high throughput liquid media dilution cultivation. There are two entries for *Lysinimonas*, which refer to two distinct species within this genus. Colours reflect the media where members of these species could be isolated.

All the species observed were classified taxonomically against the SILVA database, which allowed the selection of potentially novel bacteria. For these, the near full length 16S rRNA gene sequence was obtained and their closest relative was determined using the EzBioCloud identification tool (Yoon *et al.* 2017) (Supplementary Table 5). 97% sequence similarity was used as threshold to identity novel isolates. This confirmed the isolation of 17 novel bacteria, which represented 21.8% of the total of species isolated. Most of the novel bacteria represent novel species of known genera, with only 7 isolates representing new genera (Table 11). The highest amount of novel isolates was obtained with SSE/C-mix (10), followed by SSE/HD1:10 (5) and BEX/HD1:10 (2). No novel bacteria were isolated from the SSE/Polymermix medium. The novel isolates belong to the *Acidobacteria*, *Actinobacteria* and *Bacteroidetes* phyla and to the proteobacterial classes *Alpha-*, *Beta-* and *Deltaproteobacteria* (Figure 60).

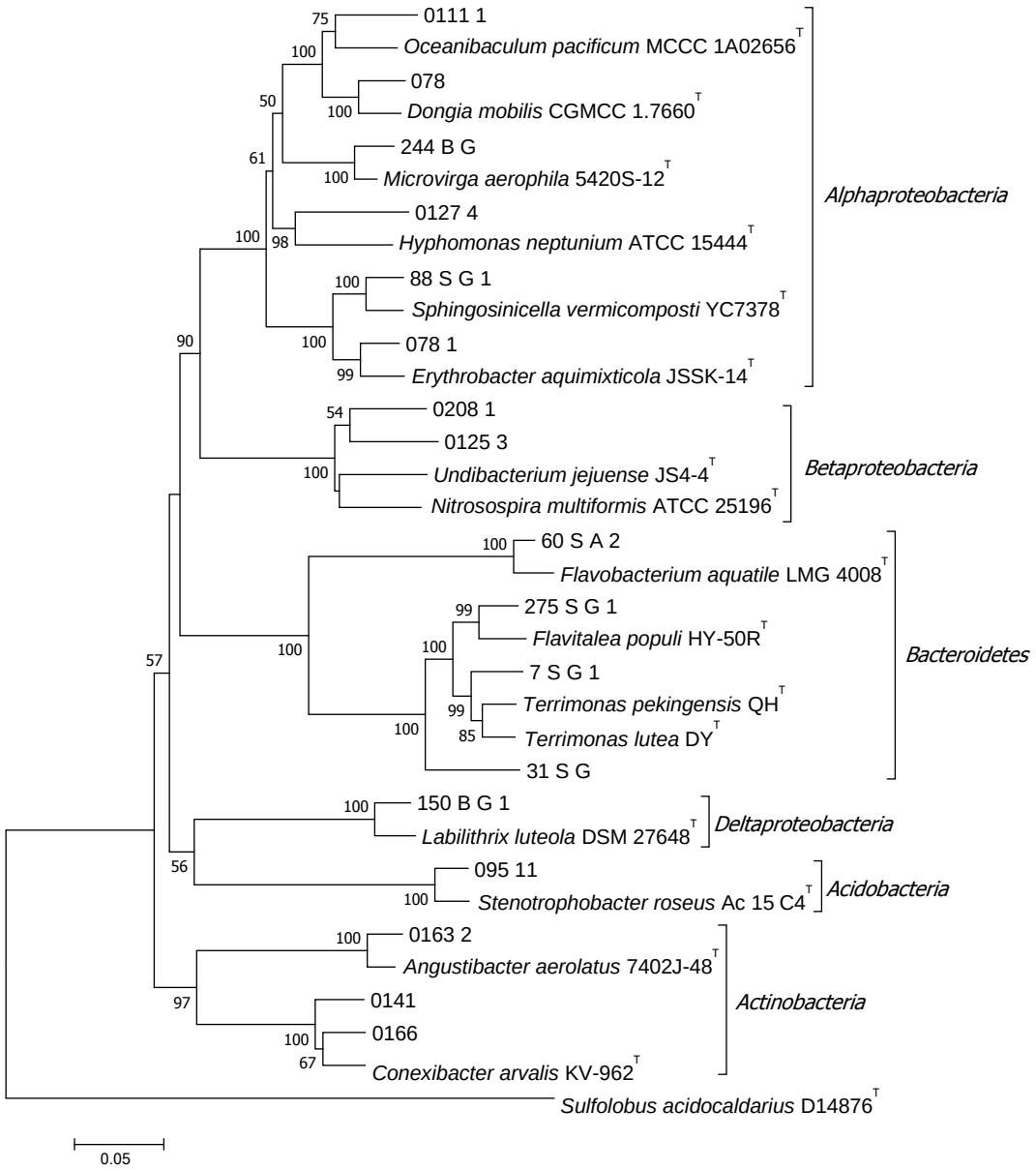


Figure 60 – Neighbour-joining tree of the novel isolates and closest validly described relatives in the high throughput liquid media dilution cultivation, based on almost full-length 16S rRNA gene sequences. The archaeal *Sulfolobus acidocaldarius* D14876 was used as the outgroup. Bar, 5 % nucleotide divergence. Only bootstrap values above 50 % are indicated at the branches (1000 replicates).

6.1.3 – Targeting biofilm forming bacteria

In order to expand the coverage of the cultivation effort, 4 inert surfaces were added to liquid SSE/HD1:10 media, in an attempt to target biofilm forming bacteria whose growth may be stimulated by the presence of a solid matrix. As for the high throughput liquid media dilution approach, after plating and incubation the partial sequences of the 16S rRNA gene were obtained. Again, after dereplicating the obtained sequences, proportional distances were calculated based on pairwise deletion and the sequences were grouped, using 97% sequence similarity as a threshold to determine different bacterial species. A total of 47 distinct species were isolated, but all were assigned to existing validly described species. The highest number of species were isolated from steel (22), followed by polystyrene (20), polypropylene (18) and glass (15) (Table 12).

Table 12 – Isolated bacteria from the different solid surfaces on the biofilm targeting approach.

Surface	Nº of isolated colonies	Nº of species
Polypropylene	46	18
Polystyrene	44	20
Glass	34	15
Steel	50	22

Although the majority of bacterial species were isolated from a specific surface, 15 were found in more than one surface type, being that 4 were present in all (Figure 61A). Regarding the taxonomy of the isolated colonies, the majority belonged to the genera *Pseudomonas*, *Bacillus* and *Flavobacterium*, regardless of surface type (Figure 61B). Nevertheless, the distribution of these seemed to vary by surface type, although the differences observed were not statistically significant (low number of replicates and different sampling effort). *Pseudomonas* seemed more prevalent in polypropylene, *Bacillus* more abundant in glass and *Flavobacterium* more enriched in polystyrene and steel. Some of the less frequently retrieved genera showed some surface specificity, such as *Ensifer* which was only found in the plastics (Figure 61B).

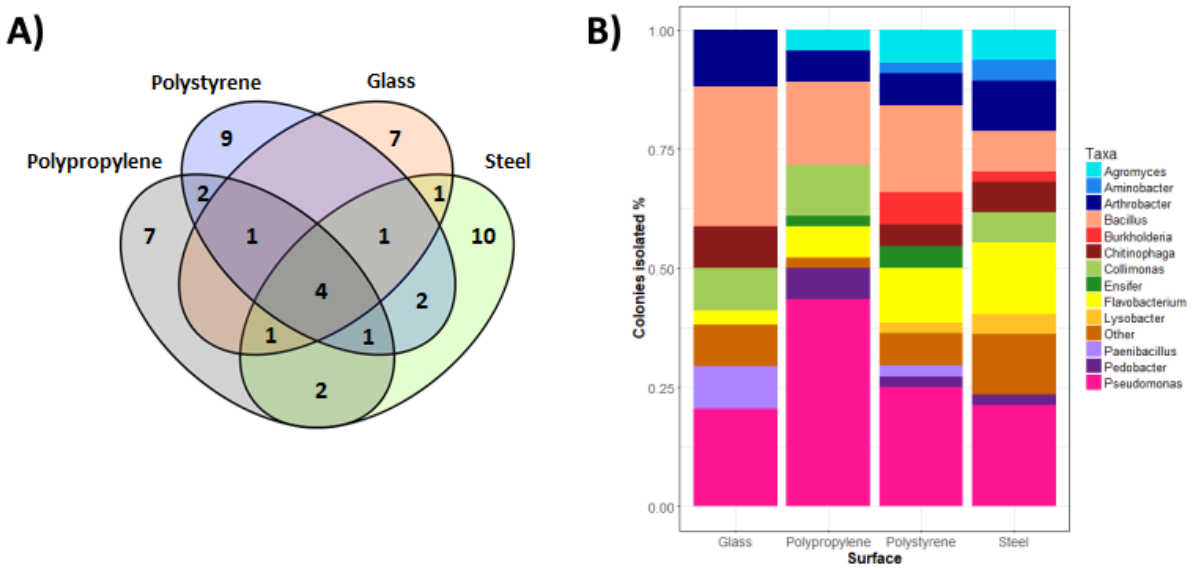


Figure 61 – Distribution and taxonomy of bacterial species growing in the distinct surfaces from the biofilm targeting approach. A) Venn diagram depicting the shared bacterial species isolated from the 4 different surfaces. B) Taxonomy at genus level of the isolated colonies by surface, based on partial 16S rRNA gene sequences blasted against the SILVA database.

Rarefaction was plotted for the distinct species isolated from the different surfaces (Figure 62). Sample coverage estimates were of 42.4%% for steel, 41% for glass, 37.9% for polypropylene and 25.4% for polystyrene. This has shown that for all surfaces it was not possible to retrieve in isolation the majority of culturable species and therefore further cultivation efforts with these conditions would yield much more diversity.

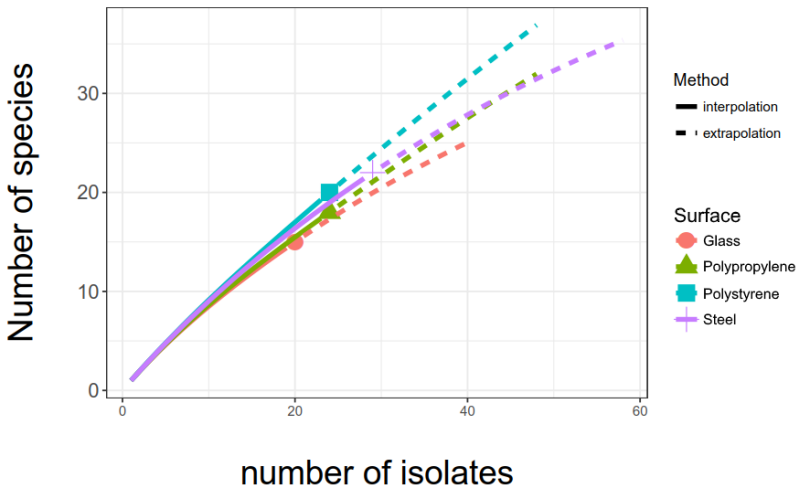


Figure 62 – Rarefaction curves for the isolated bacterial species from the four different surfaces used in the biofilm targeting approach.

6.1.4 – Direct plating

Bacterial interactions are an important factor to consider when attempting to isolate these organisms in pure culture. Due to this, a direct plating approach was employed in order to target bacteria which produce (micro-) colonies in solidified media and which growth may be supported by neighbouring organisms. Three different dilutions of the soils were plated in SSE/HP media solidified with gellan gum and submitted to a long incubation period. Distinct samples yielded distinct results, with samples HEW09, SEG01 and SEW02 producing generally lower culturability values (Table 13), which is consistent with the results obtained in the high throughput liquid cultivation dilution approach for culturability (Figure 48). Interestingly, at the highest dilution these samples showed improved culturability, a trend not verified for the remaining samples.

Table 13 – Number of inoculated cells, visible growing colonies and percentage of culturability after incubation in the direct plating plates, by dilution.

	10^{-6}			10^{-7}			10^{-8}		
Samples	**Nº cells inoculated**	**Nº visible colonies**	**Culturability %**	**Nº cells inoculated**	**Nº visible colonies**	**Culturability %**	**Nº cells inoculated**	**Nº visible colonies**	**Culturability %**
AEG08	2530	80	3.16	253	8	3.16	25.3	0	0
AEW08	3324	115	3.46	332.4	27	8.12	33.2	0	0
HEG06	3044	113	3.71	304.4	13	4.27	30.4	0	0
HEW09	3270	8	0.24	327	4	1.22	32.7	21	64.22
SEG01	3590	57	0.7	359	3	0.84	35.9	2	5.57
SEW02	2680	13	0.49	268	0	0	26.7	1	3.75

Partial 16S rRNA gene sequences were determined for all picked colonies and were later dereplicated. As before, proportional distances were calculated, and sequences were grouped based on 97% sequence similarity. The 266 colonies evaluated represented a total of 107 distinct species (Table 14). Regarding the taxonomy of the isolated colonies, the majority belonged to the *Flavobacteriaceae*, *Sphingomonadaceae* and *Cytophagaceae* (Figure 63).

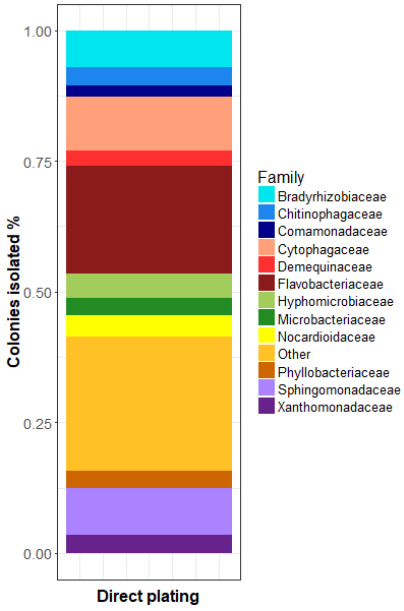


Figure 63 –Taxonomy at family level of the isolated colonies with the direct plating approach, based on partial 16S rRNA gene sequences blasted against the SILVA database.

Many of the species (32%) revealed similarity values lower than 97% to any known validly described bacteria (Table 14). This was further verified by amplification of the near full length 16S rRNA gene sequence and subsequent determination of the closest relative using the EzBioCloud identification tool (Yoon *et al.* 2017) (Supplementary Table 6). 97% sequence similarity was used as threshold to identity novel isolates. In contrast to the high throughput liquid cultivation dilution approach, most of the novel bacteria represent novel genera (a few possibly represent even higher taxonomic ranks) (70.6%), with only 10 (29.4%) representing novel species of known genera.

Table 14 – Number of inoculated cells and number of visible growing colonies after incubation in the direct plating plates, by dilution.

Samples	Nº of isolated colonies	Nº of species	Nº of novel isolates < 97% 16S similarity	Nº of novel isolates < 95% 16S similarity	Nº of novel isolates < 90% 16S similarity	Total nº of new isolates
AEG08	45	16	2	1	0	3
AEW08	100	28	6	4	0	10
HEG06	67	37	3	8	3	14
HEW09	19	17	2	0	0	2
SEG01	31	20	0	7	1	8
SEW02	4	3	0	0	0	0

The novel isolates belonged to 8 different bacterial phyla, with some representing novel members of underexplored phyla such as *Acidobacteria*, *Armatimonadetes* and *Chloroflexi* (Figure 64). Moreover, the planctomycetal AEW_-6_35 represented the first isolate of its phylum originating from soil.

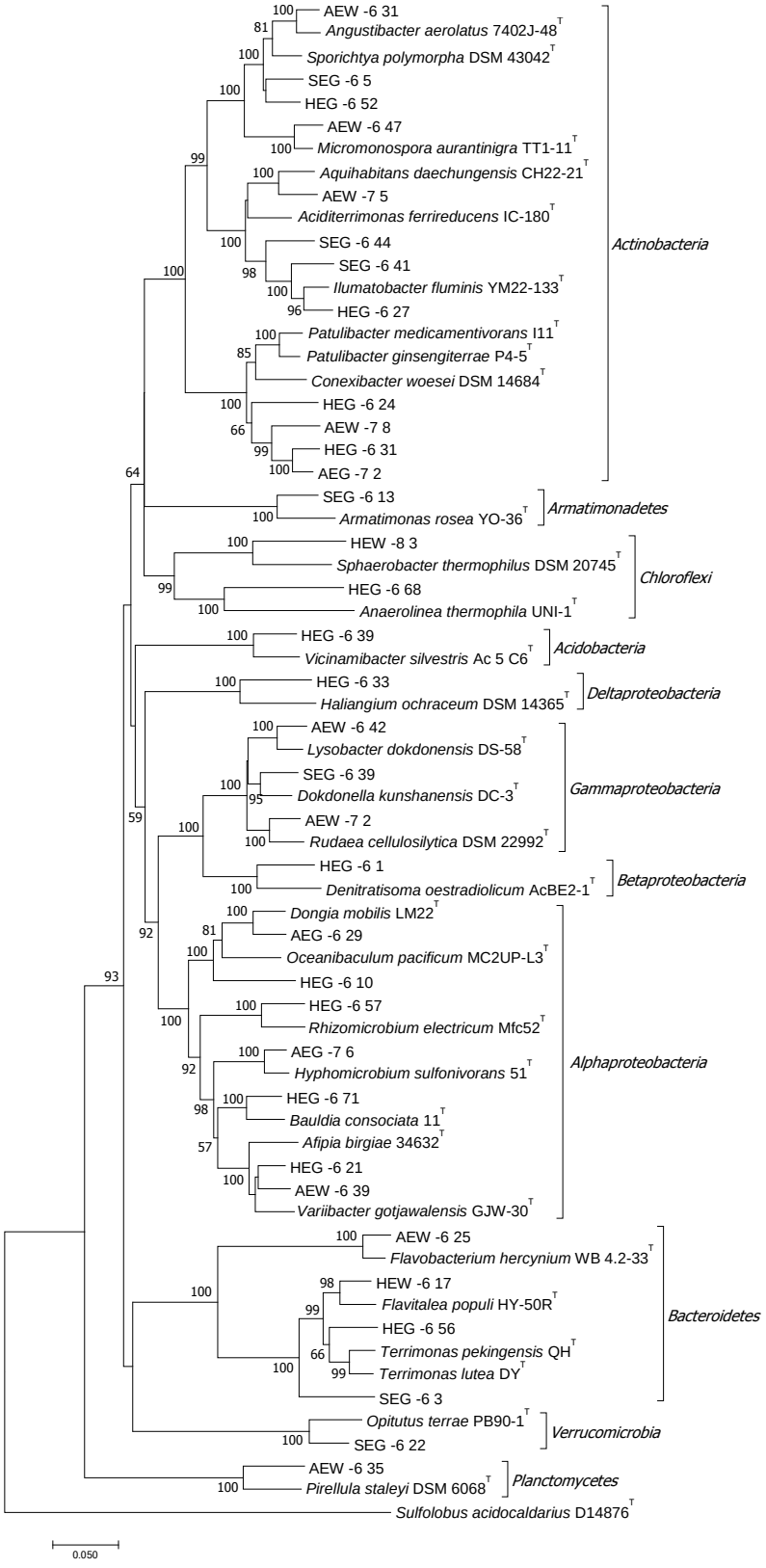


Figure 64 – Neighbour-joining tree of the novel isolates and closest validly described relatives the direct plating cultivation approach, based on almost full-length 16S rRNA gene sequences. The archaeal *Sulfolobus acidocaldarius* D14876 was used as the outgroup. Bar, 5 % nucleotide divergence. Only bootstrap values above 50 % are indicated at the branches (1000 replicates).

6.1.5 – Output of different approaches

The different approaches employed generally allowed the isolation of different bacteria (Figure 65). Nevertheless, some bacterial species were recovered with two or even all approaches. Most of the shared species occurred between the high throughput liquid medium dilution and direct plating approaches. Even though the medium used in the biofilm targeting approach was also used in the high throughput liquid media dilution (SSE/HD1:10), the seven species isolated with both approaches didn't necessarily arise from this specific medium.

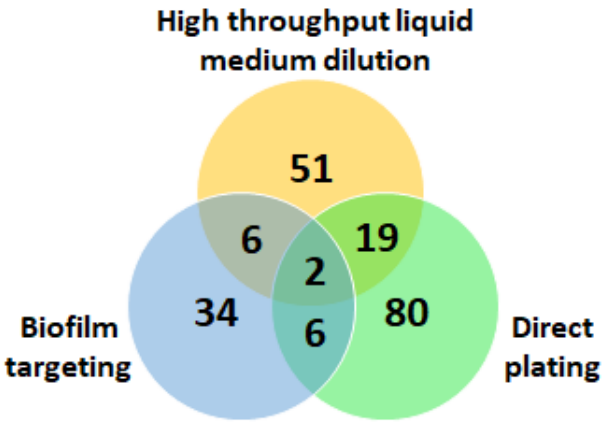


Figure 65 – Venn diagram depicting the shared bacterial species isolated from the 3 different cultivation approaches.

Rarefaction was calculated for all isolated species from all the tree different cultivation approaches and it was evident that more species can still be retrieved from the environment tested when using these cultivation approaches (Figure 66). Sample coverage estimates were of 68.5% for the high throughput liquid medium dilution, 66% for the biofilm targeting approach and 22.3% for direct plating. Not only was the direct plating the approach which effectively retrieved a higher number of species, but it was also the one with the biggest potential of retrieving even more, since 77.7% of all the estimated species which potentially could be isolated with this medium still eluded cultivation.

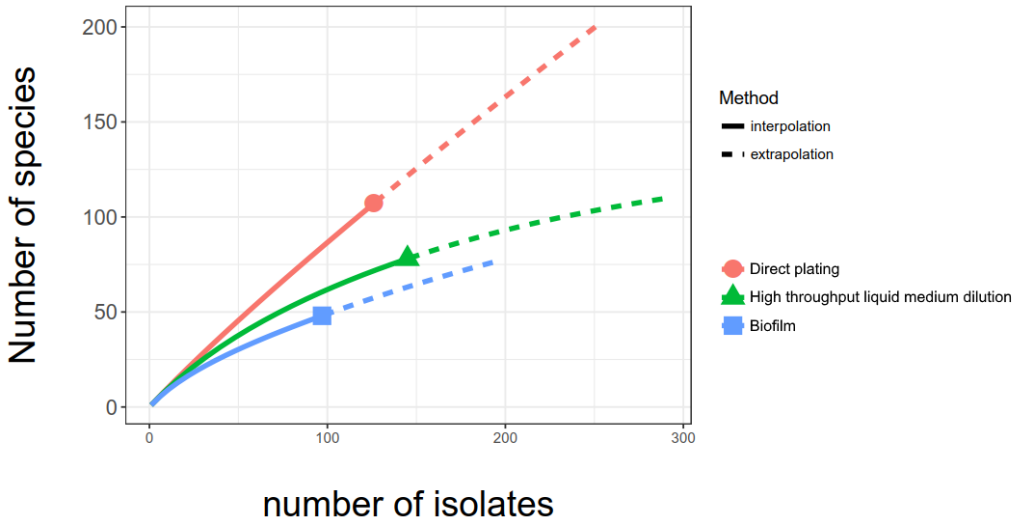


Figure 66 – Rarefaction curves for the bacterial species isolated with the three different cultivation approaches.

Even though the highest number of picked colonies was obtained with the high throughput liquid medium dilution approach, the highest amount of species was isolated with the direct plating approach (Table 15), which was also reflected on the number of novel isolates. The biofilm targeting approach yielded the least amount of isolates, none of which was novel. Between the two approaches that generated novel isolates, the direct plating approach allowed the isolation of more strains with lowest similarity values (lower than 95% sequence similarity with validly described bacteria). Although most of the novel bacterial strains were specific to the approach from which they derived, two isolates were retrieved from both the direct plating and the high throughput liquid medium dilution approaches.

Table 15 – Number of inoculated cells and number of visible growing colonies after incubation in the direct plating plates, by dilution.

Approach	**Nº of isolated colonies**	**Nº of species**	**Nº of novel isolates < 97% 16S similarity**	**Nº of novel isolates < 95% 16S similarity**	**Total nº of new isolates**
High throughput liquid medium dilution	406	78	12	5	17
Biofilm targeting	171	48	0	0	0
Direct plating	266	107	11	23	34

Although the novel isolates were derived from just 8 of the 47 bacterial phyla which were present in the cultivated soil samples, more diverse strains were isolated with the direct plating approach (Figure 67), Including members of rarely isolated groups such as *Armatimonadetes* and *Chloroflexi*.

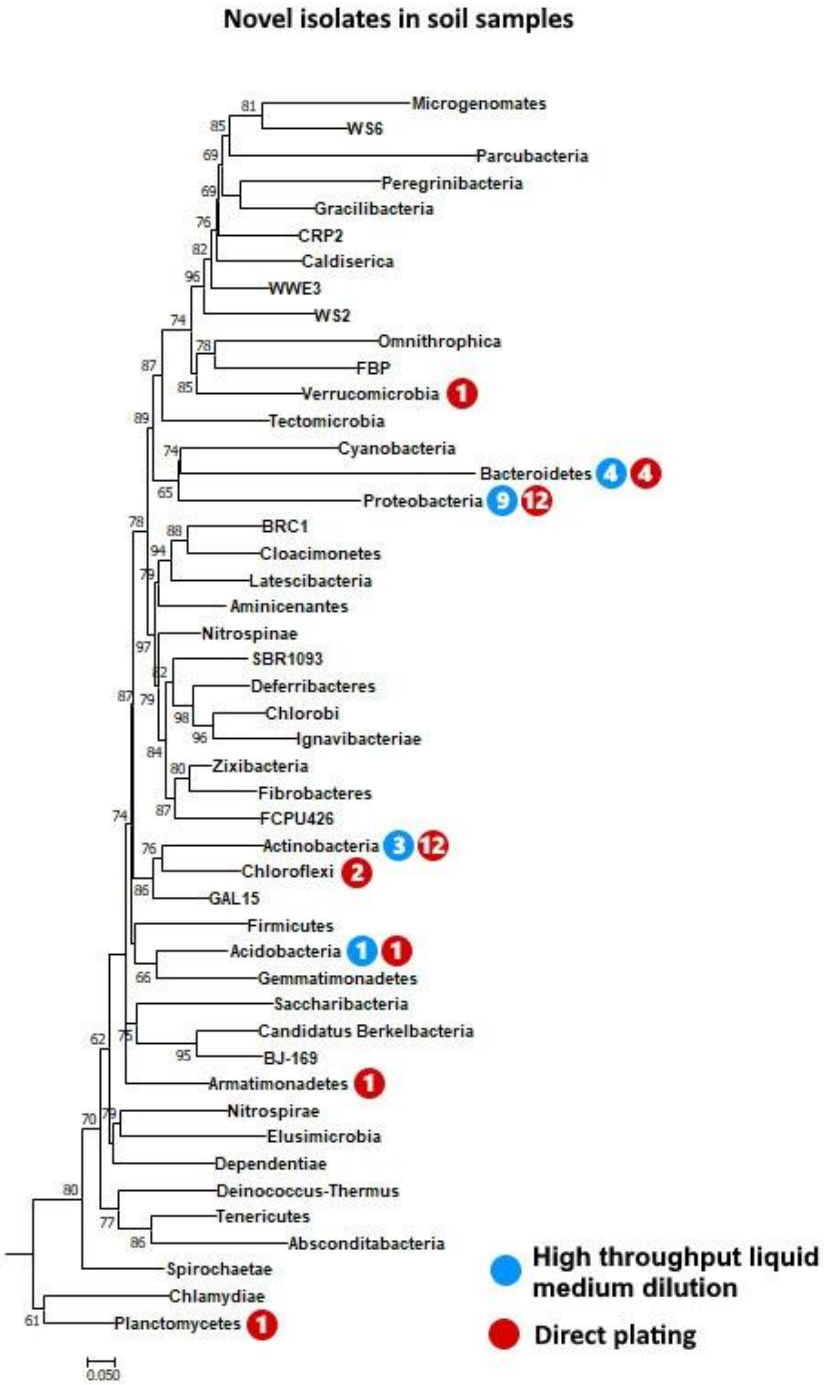


Figure 67 – Approximately-maximum-likelihood phylogenetic (Fastree) inferred from V3 amplicons from the six soil samples used for cultivation, agglomerated at phylum level. Circles mark bacterial phyla for which the different cultivation approaches yielded novel strains. The number inside the circles reveal the amount of novel isolates isolates. Bar, 5 % nucleotide divergence.

6.1.6 – Direct plating method on sample of a different soil environment

Since the direct plating approach yielded the best results in terms of number and diversity of novel bacteria isolated with the grassland and forest soil samples, this same approach was tested again. The sample used was collected in the immediate surrounding of a swamp and was chosen because it portrayed a very different soil environment as the previous utilized samples. This therefore allowed to determine the reproducibility of this approach and also its applicability in soil environments in general.

The partial 16S rRNA gene sequences from all the 174 colonies obtained were dereplicated and the proportional distances were calculated. A total of 88 different species were present, based on the grouping of the sequences based on 97% sequence similarity (Table 16).

Table 16 – Number of inoculated cells and number of visible growing colonies after incubation in the direct plating plates with the swamp sample.

Nº of isolated colonies	Nº of species	Nº of novel isolates < 97% 16S similarity	Nº of novel isolates < 95% 16S similarity	Nº of novel isolates < 90% 16S similarity	Total nº of new isolates
174	88	10	15	5	30

When comparing the direct plating method between the two distinct soil environments (swamp versus grassland and woodland soils), it was evident that the majority of isolated species was specific to the sample origin. Only 13 species were obtained from both environments (Figure 68).

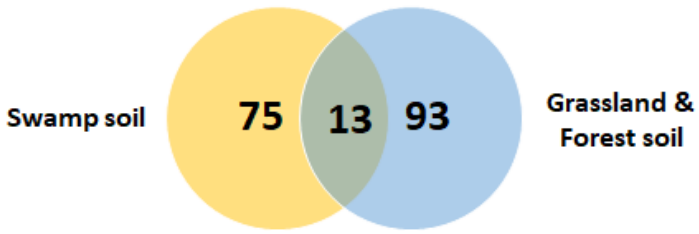


Figure 68 – Venn diagram depicting the shared bacterial species isolated from the 2 different soil environments with the direct plating approach.

Regarding the taxonomy of the isolated colonies, the majority belonged to the *Cytophagaceae*, *Comamonadaceae* and *Chitinophagaceae* (Figure 69).

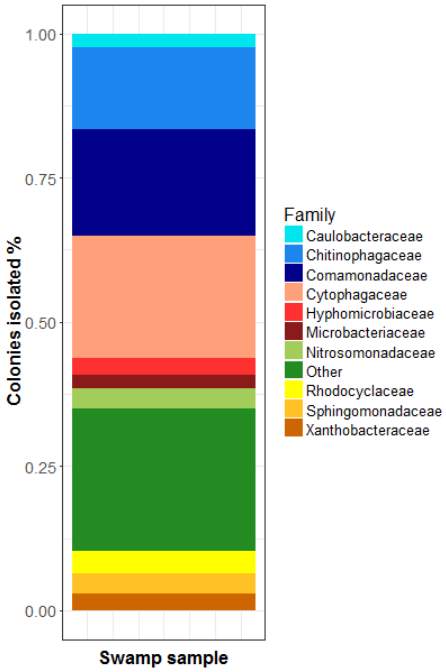


Figure 69 –Taxonomy at family level of the isolated colonies with the direct plating approach for the swamp sample, based on partial 16S rRNA gene sequences blasted against the SILVA database.

As was the case for the bacteria cultivated from grassland and woodland samples with the same method, approximately a third of the (34.1%) of the species isolated were novel (Table 16). This was further verified by amplification of the near full length 16S rRNA gene sequence and subsequent determination of the closest relative using the EzBioCloud identification tool (Yoon *et al.* 2017) (Supplementary Table 7). Two thirds of the novel bacteria represent novel genera (or even higher taxonomic ranks), with only a third representing representing novel species of known genera. This is also quite similar to the results from grassland and woodland samples (70.6% novel genera or higher, 29.4% novel species of known genera).

The novel isolates belonged to 6 different bacterial phyla, with some representing novel members of underexplored phyla such as *Gemmatimonadetes* and *Chloroflexi* (Figure 70).

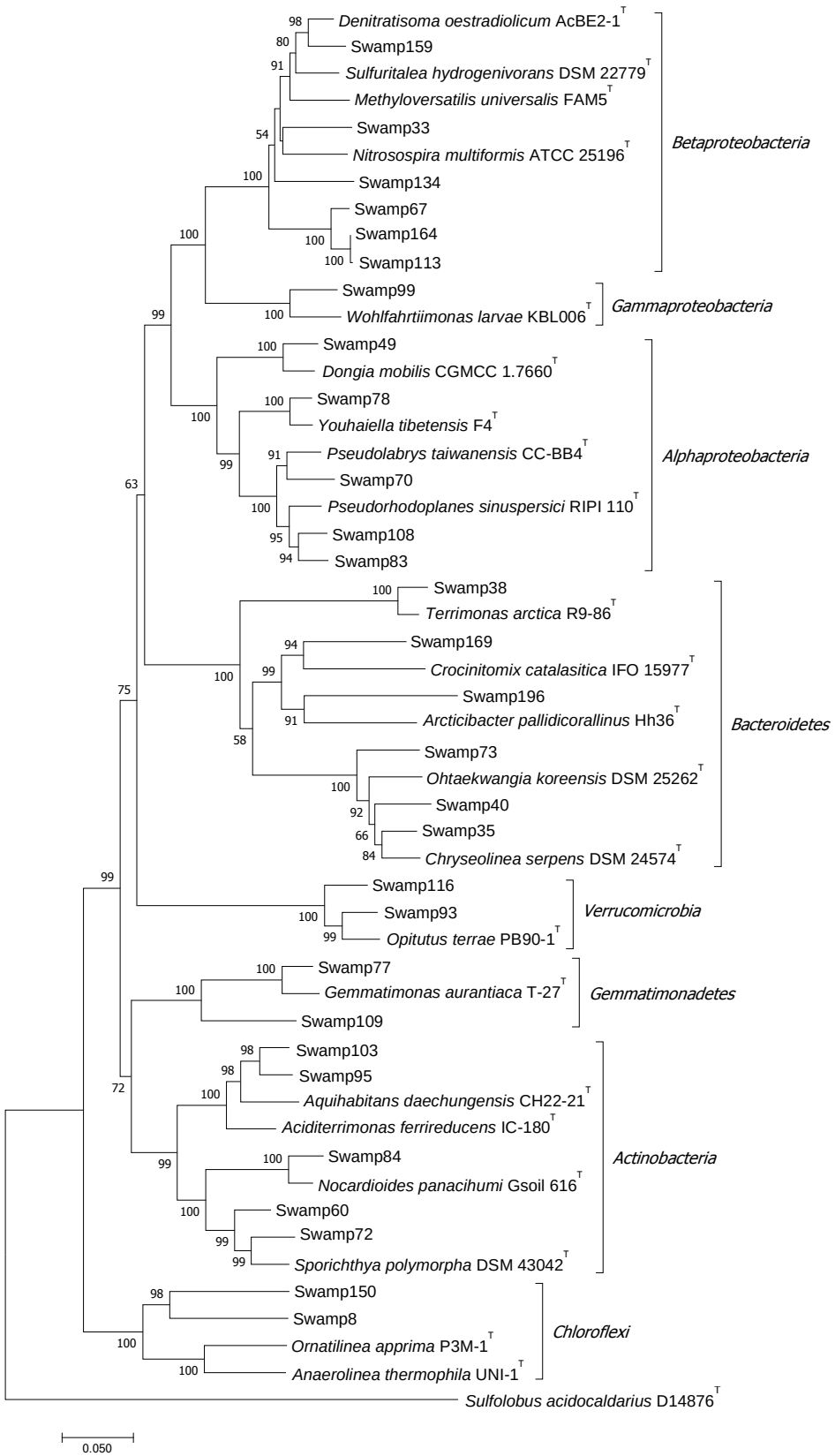


Figure 70 – Neighbour-joining tree of the novel isolates the direct plating cultivation approach for the swamp sample, based on almost full-length 16S rRNA gene sequences. The archaeal *Sulfolobus acidocaldarius* D14876 was used as the outgroup. Bar, 5 % nucleotide divergence. Only bootstrap values above 50 % are indicated at the branches (1000 replicates).

6.1.7 – Novel isolates in various soil environments

To evaluate the importance of all the novel isolates (defined as less than 97% full length 16S rRNA gene sequence similarity to a validly described strain and summarized in the phylogenetic tree of Figures 71-73) in their original environment, their full 16S rRNA gene sequence was analysed by BLAST against the V3 amplicons of the soil samples used for cultivation (Figure 71). Most of the novel strains isolated were rare in their environment, especially in sample SEW02 (forest sample of the Schorfheide-Chorin region). Nevertheless, some were abundant, as the actinobacterial strains SEG_-6_41 and HEG_-6_27 and the alphaproteobacterial HEG_-6_21, 244_B_G_1 and HEG_-6_71.

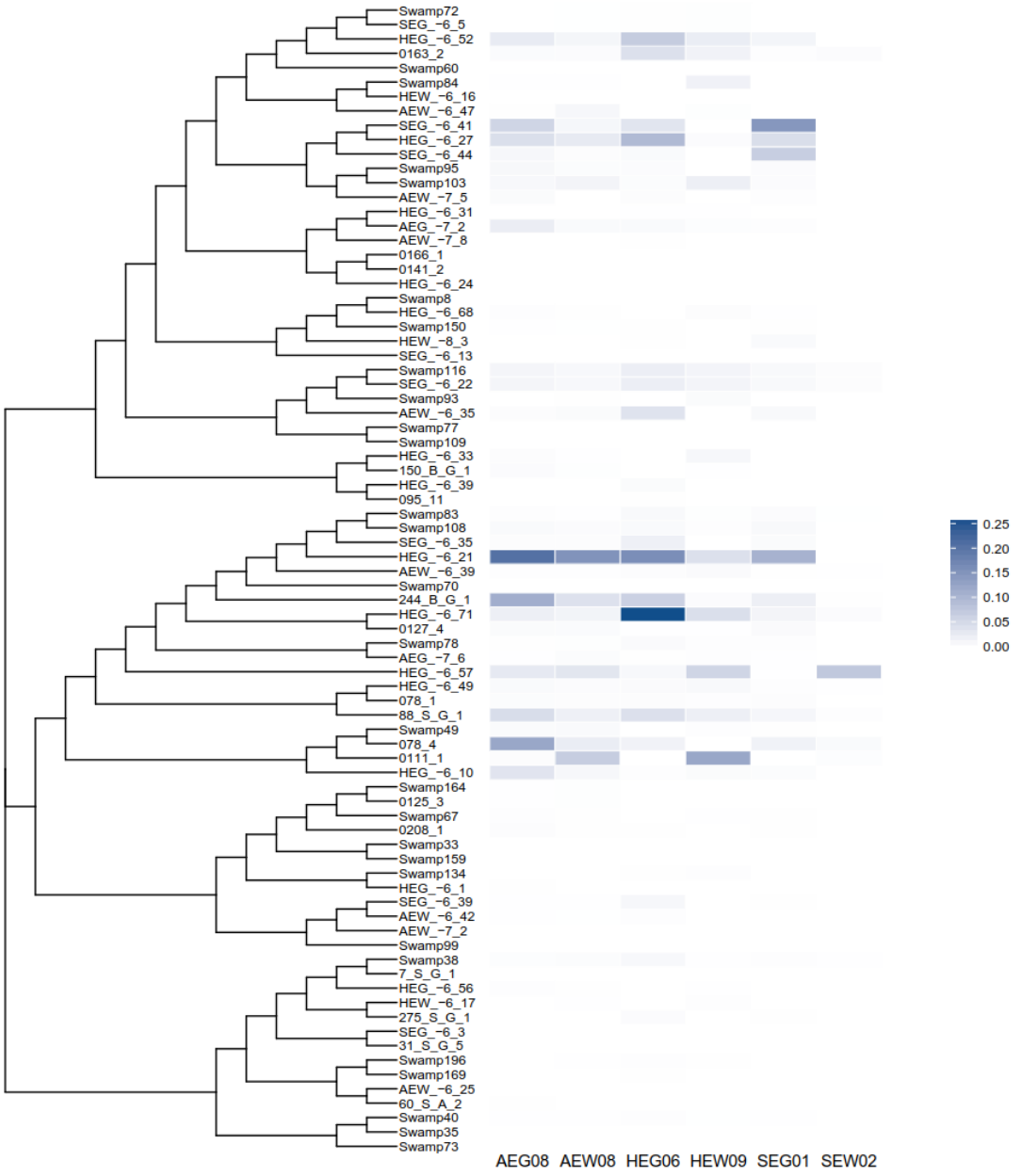


Figure 71 – Neighbour-joining tree of the novel isolates based on almost full-length 16S rRNA gene sequences, with heatmap showing their relative abundances on the original soil samples.

In the same fashion, to assess the importance of the novel isolates in other soil environments other than the ones where the bacteria originated, their full 16S rRNA gene sequence was analysed by BLAST against the amplicon datasets from rhizosphere and from the mineral colonization experiments (Chapters 4 and 5).

The majority of the novel isolates were rare in the rhizosphere environment, but also in the corresponding control bulk soil. Nevertheless, some of the strains were abundant, especially members of *Actinobacteria* and *Alphaproteobacteria*. The most abundant isolate in this environment was 88_S_G_1, a possible novel species of the *Sphingosinicella* genus. For some isolates such as 0163_2, HEG_-6_27, HEG_-6_49 and 88_S_G_1 it was possible to observe the rhizosphere effect, with relative abundances being generally higher in the rhizosphere samples as opposed to bulk soil (Figure 72).

As for the rhizosphere environment, most of the novel isolates were also rare on the mineral colonization experiment (Figure 73). The most abundant isolate in this environment, as was the case for the rhizosphere, was 88_S_G_1, which becomes abundant in both minerals and roots over time. Other alphaproteobacterial isolates such as HEG_-6_49 and HEG_-6_57 are also abundant and show differential abundance, with the first being more prevalent in the minerals of the simple carbon addition experiment and the second being enriched in the minerals and roots of the complex carbon experiment. The verrucomicrobial isolates Swamp116 and SEG_-6_22 also became abundant at the later stages in the minerals and roots but only of the complex carbon experiment. As opposed to the rhizosphere environment, the novel bacterial isolates from *Actinobacteria* did not indicate a big role in this environment.

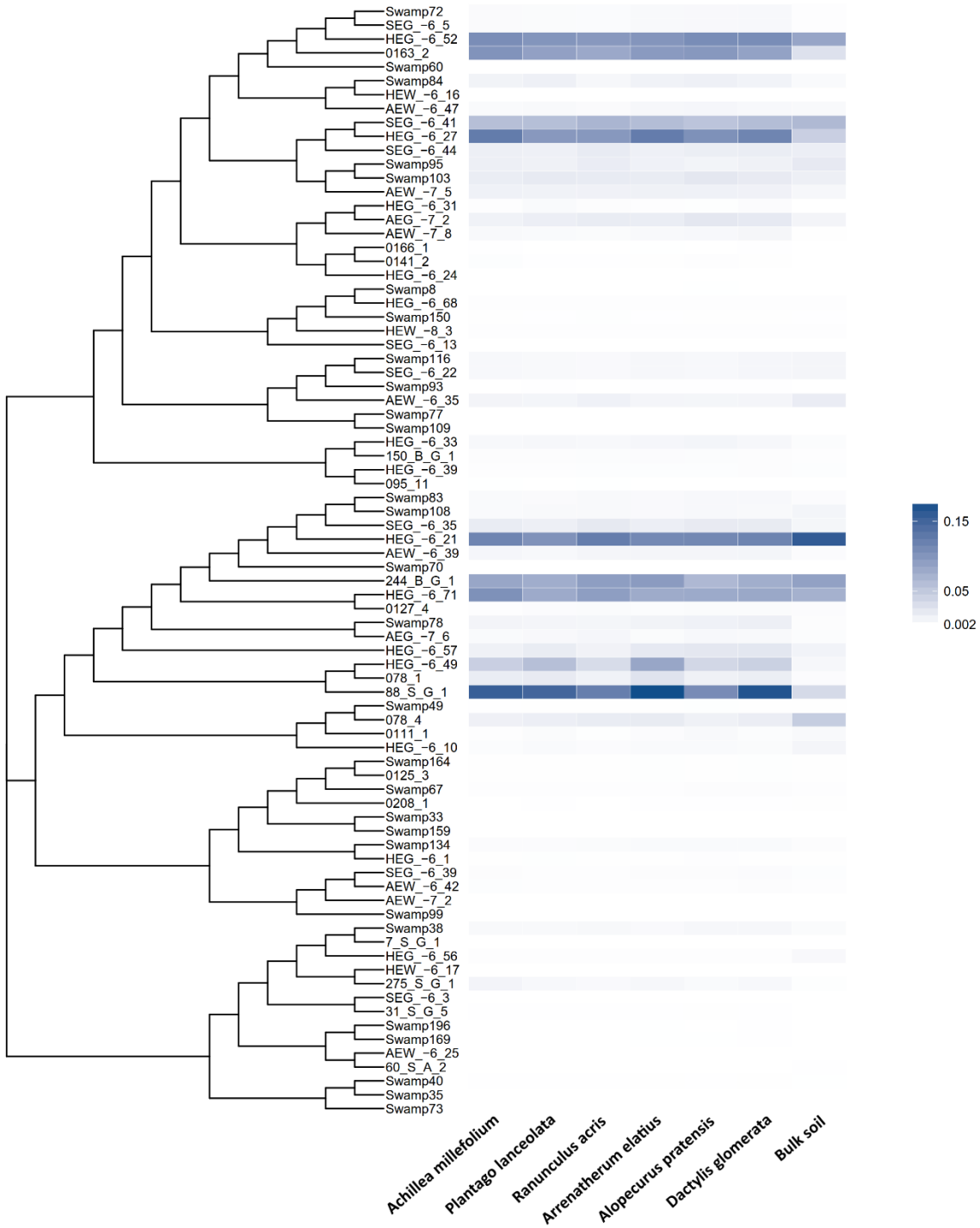


Figure 72 – Neighbour-joining tree of the novel isolates based on almost full-length 16S rRNA gene sequences, with heatmap portraying the relative abundances of the novel isolates on the rhizosphere amplicon dataset from Chapter 4.

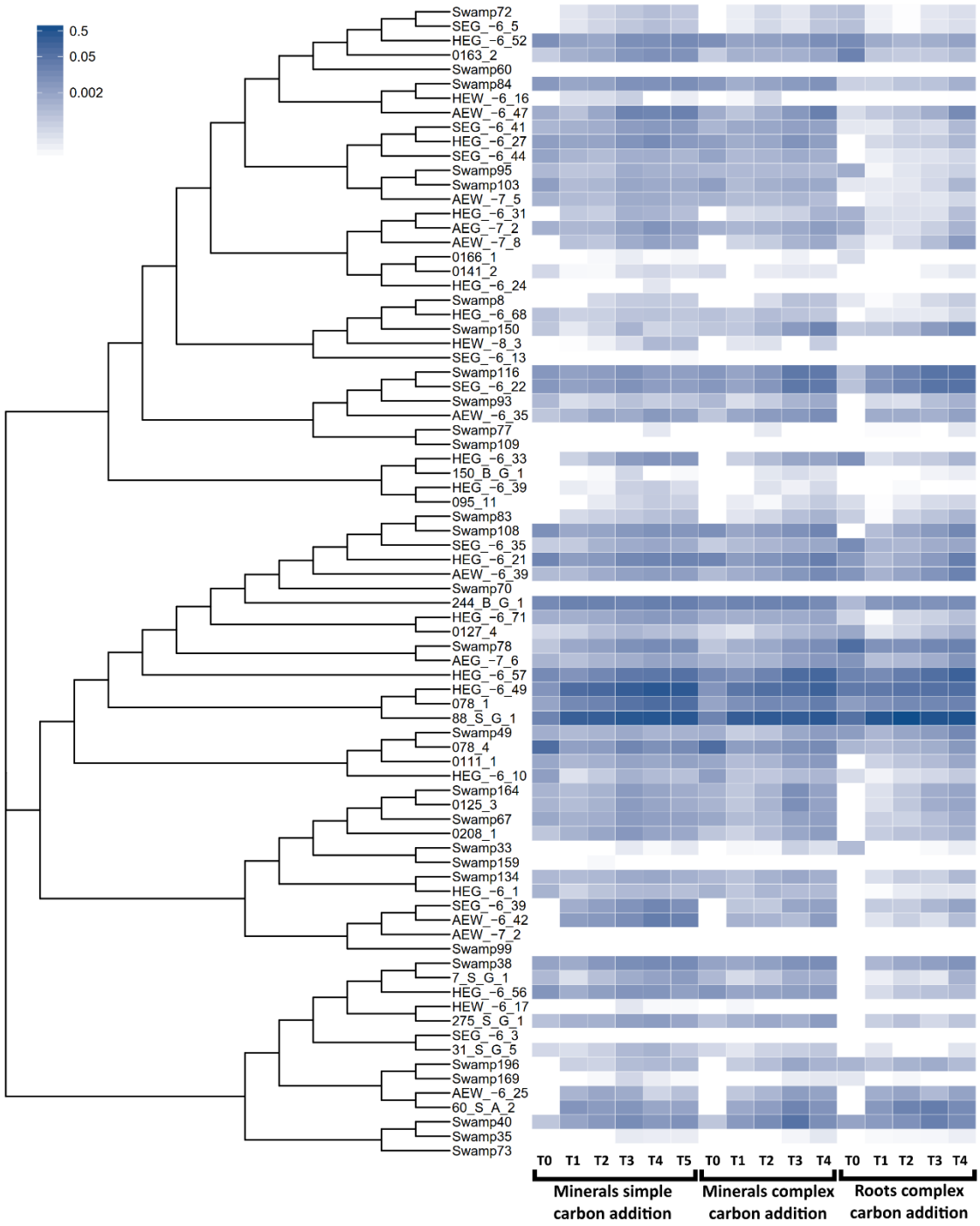


Figure 73 – Neighbour-joining tree of the novel isolates based on almost full-length 16S rRNA gene sequences, with heatmap portraying the relative abundances of the novel isolates on the mineral colonization amplicon datasets from Chapter 5. Colour scale is logarithmic.

6.1.8 – Chemotaxonomic and genomic investigation of *Acidobacteria* subdivision 6 strain HEG_-6_39^T *Luteitalea pratensis* gen. nov. sp. nov.

Strain HEG_-6_39^T was selected for further characterization, chosen due to their taxonomic novelty and readiness to grow in laboratory conditions. Despite not being very abundant in the soil from which it originated, HEG_-6_39^T is abundant in (mainly soil) environments worldwide (Table 17). This bacterium, named *Luteitalea pratensis*, represented a novel genus of subdivision 6 (sd6) *Acidobacteria* and has been validly described and published (Vieira *et al.* 2017).

Table 17 – Environments were HEG_-6_39^T is most abundant, obtained by blast of the 16S rRNA gene sequence against the 16S rRNA gene amplicon-based microbial profiles of imngs depository, at 99% similarity (Lagkouvardos *et al.* 2016).

Description	Location	Relative abundance %
Dry mineral soils associated with snow patches	Taylor and Wright Valleys, Antarctica	3.00
Dry mineral soils associated with snow patches	Taylor and Wright Valleys, Antarctica	2.09
Dry mineral soils associated with snow patches	Taylor and Wright Valleys, Antarctica	1.56
Arable soil with Medicago-topsoil	Germany	1.50
Agricultural soil	Shannxi, China	1.44
Artificial plant growth substrate	Prague, Czech Republic	1.29
Dry mineral soils associated with snow patches	Taylor and Wright Valleys, Antarctica	1.23
Dry mineral soils associated with snow patches	Taylor and Wright Valleys, Antarctica	1.21
Rhizosphere of switchgrass (*Panicum virgatum* L.)	Not specified	1.04
Arable soil with Medicago-topsoil	Germany	0.98

Despite of the steadily growing number of *acidobacterial* 16S rRNA gene sequences, the cultured diversity of this phylum is still very limited. Cultivation success so far is strongly biased towards sd1 *Acidobacteria*, to which the majority of isolates are affiliated. In opposition, the first and only other representative of sd6, *Vicinamibacter silvestris* Ac_5_C6 ^{T} was only very recently described (Huber *et al.* 2016). The characterization of a novel isolate of this subdivision will therefore enhance our knowledge of this group, which is important since sd6 represents the most numerous and phylogenetically diverse *acidobacterial* subdivision (George *et al.* 2011).

Strain HEG_-6_39^T was isolated from a mown pasture grassland soil in Thuringia (central eastern Germany) located close to Mühlhausen, Germany (N 51°12’53.971”, E 10°23’27.992”) and was characterized by a polyphasic approach. This strain formed round, convex, dark yellow colonies with entire margins that reached 0.5-1.0 mm in diameter within 10 days of growth at 28°C. Light microscopic observations revealed that the cells divided by binary fission and were rod shaped, 0.9-

1.4 µm long and 0.6-0.75 µm in diameter. The strain stained Gram-negative and was non-spore-forming, similar to described members of the *Acidobacteria* phylum. Capsules could not be detected like for *Vicinamibacter silvestris* Ac_5_C6 ^{T} (Huber *et al.* 2016), the only other validly described member of sd6 (Table 7). The ultrastructural analyses confirmed the Gram-negative cell-wall structure of strain HEG_-6_39^{T} and revealed high amounts of ribosomes and intracellular inclusion bodies (Figure 74).

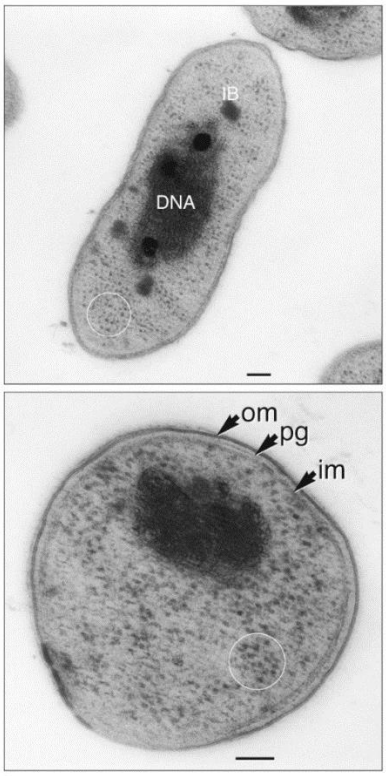


Figure 74 – Transmission electron micrographs of an ultrathin section of strain HEG_-6_39^{T}; scale bar, 100 nm; IB = inclusion body; circle = ribosomes: om = outer membrane; im = inner membrane; pg = peptidoglycan.

Catalase activity was determined and strain HEG_-6_39^{T} tested positive in accordance with the majority of acidobacterial isolates. Unlike *Vicinamibacter silvestris* Ac_5_C6 ^{T}, the novel isolate tested negative for cytochrome *c*-oxidase activity (Table 18).

The relationship of the strain HEG_-6_39^{T} to oxygen was assessed, confirming the strain to be an aerobe. Growth ranges and optima pH were determined, and growth was observed from pH 5.3 to 8.3 (optimal pH 6.1 - 7.5). Optimum temperatures for growth were between 20 and 30°C, though strain HEG_-6_39^{T} was capable of slow growth at temperatures between 0 to 36°C. Compared to *Vicinamibacter silvestris* Ac_5_C6^{T}, it tolerated a narrower range of pH, but it could grow at much

lower temperature values (Table 18). Similar to most *Acidobacteria*, the new isolate tolerated NaCl concentrations up to 1%, although it grew best at concentrations between 0 and 0.25% (Table 18). Doubling times under optimal conditions were 29.8 h, which is significantly slower than the majority of acidobacterial isolates to date.

Table 18 – Differential characteristics of HEG_-6_39^T compared with the type strains of the closest *Acidobacteria* genera. Strains: 1, HEG_-6_39^T; 2, *Vicinamibacter silvestris* Ac_5_C6^T(Huber *et al.* 2016),3, *Acanthopleuribacter pedis* FYK2218^T(Fukunaga *et al.* 2008); 4, *Geothrix fermentans* H-5^T (Coates *et al.* 1999); 5, *Holophaga foetida* TMBS4^T (Liesack *et al.* 1994); 6, 'Thermotomaculum hydrothermale' AC55^T (Izumi *et al.* 2012); 7, *Thermoanaerobaculum aquaticum* MP-01^T (Losey *et al.* 2013); +, positive; -, negative; ND, no data available.

Characteristics	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Cell shape	rod	rod	rod	rod	rod	rod	rod
Cell length/width (µm)	0.9-1.4 0.6-0.75	1.3-2.0 0.6-0.7	2.4-4.7 0.7-1.0	1.0-2.0 0.1	1.0-3.0 0.5-0.7	2.0-6.8 0.3-0.6	3.5-8.0 0.2-0.3
Motility	-	-	+	-	-	-	-
Pigmentation	dark yellow	yellow	yellow	white	translucent-beige	ND	ND
Spores	-	-	ND	-	ND	ND	ND
Capsule/	-	-	ND	ND	ND	ND	ND
Oxidase	-	+	+	ND	+	ND	ND
Catalase	+	+	+	ND	ND	ND	ND
NaCl [% (w/v)]	≤1.0	≤1.0	ND	ND	ND	1.5-4.5	≤0.5
Temperature for growth (°C)							
Range	0-36	12-40	15-30	ND	10-35	37-60	50-65
Optimum	20-30	26-31	30	35	28-32	55	60
pH for growth							
Range	5.3-8.3	4.7-9.0	5.0-9.0	ND	5.5-8.0	5.5-8.0	6.0-8.0
Optimum	6.1-7.5	6.5-8.1	7.0-8.0		6.8-7.5	6.6	6.5-7.0
DNA G+C-content [mol %]	64.7	65.9	56.7	68.9	62.5	51.6	62.7

The capability of strain HEG_-6_39^T to metabolize different substrates was evaluated and utilization was mostly restricted to sugars and proteinaceous substrates like peptone, yeast extract, casein hydrolysate and casamino acids (Supplementary Table 8). This is a common feature of sd1 and also of *Vicinamibacter silvestris* Ac_5_C6 ^{T}. No degradation of polymeric substrates was detected for strain HEG_-6_39^T. The pattern of exoenzyme activity for this strain was similar to its closest

described relative *Vicinamibacter silvestris* Ac_5_C6 ^{T}, though no valine arylamidase, cysteine arylamidase or gelatinase activities could be detected.

The analysis of isoprenoid quinones revealed menaquinone-8 (MK-8) to be the sole respiratory quinone in HEG_-6_39^{T} which is in accordance with the majority of the described representatives of *Acidobacteria*. The major polar lipids detected were diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG) and two unknown phospholipids (Supplementary Figure 1). With the exception of the unknown phospholipids, the composition of the polar lipids resembles that of *Aridibacter* spp. (Huber *et al.* 2014) and *Stenotrophobacter* spp. (Pascual *et al.* 2015), which are members of sd4. The polar lipid composition is also similar to the one reported for *Vicinamibacter silvestris* Ac_5_C6 ^{T}, but in the latter an unknown glycolipid was detected. The major fatty acids were iso-$C_{15:0}$ (30.3%), summed feature 3 ($C_{16:1}$ *ω6c*/ $C_{16:1}$ *ω7c*) (18.4%), $C_{18:1}$ *ω9c* (17.1%) and *iso*-$C_{17:1}$ *ω9c* (15.7%). Additionally, *iso*-$C_{17:0}$ (5.0%), $C_{18:0}$ (2.8%) and $C_{16:0}$ (2.2%) were also found in considerable amounts (Supplementary Table 9). Consistent with the majority of *Acidobacteria* species described so far and opposite to the closest relative *Vicinamibacter silvestris* Ac_5_C6^{T}, the methyl-branched fatty acid iso-$C_{15:0}$ dominates. In contrast, $C_{18:1}$ ω9c is a major fatty acid of strain HEG_-6_39^{T}, but is only found in minor amounts in all other described *Acidobacteria*, with the exception of *Acidobacterium capsulatum* 161^{T} (Kishimoto *et al.* 1991).

Susceptibility to antibiotics was assessed and strain HEG_-6_39^{T} was resistant to 21 of the 36 antibiotics tested, mainly penicillins, cephalosporins (with the exception of ceftriaxone), quinolones, carbapenems, monobactams and macrolides (Supplementary Table 10).

The 16S rRNA gene sequence accession number for strain HEG_-6_39^{T} is KT287072. Pairwise 16S rRNA gene sequence similarity calculated using p-distance revealed that strain HEG_-6_39^{T} had *Vicinamibacter silvestris* Ac_5_C6 ^{T} as its closest described relative with a similarity of 93.6%. Phylogenetic trees were calculated using neighbor-joining and maximum likelihood algorithms which placed this strain within *Acidobacteria* sd6, as a monophyletic group with *Vicinamibacter silvestris* Ac_5_C6^{T} and other bacteria isolated from a surface soil in Louvain-La-Neuve, Belgium (Figure 75; Supplementary Figure 3). The G+C content for HEG_-6_39^{T} was 64.7% as inferred from the full genome sequence (Huang *et al.* 2016).

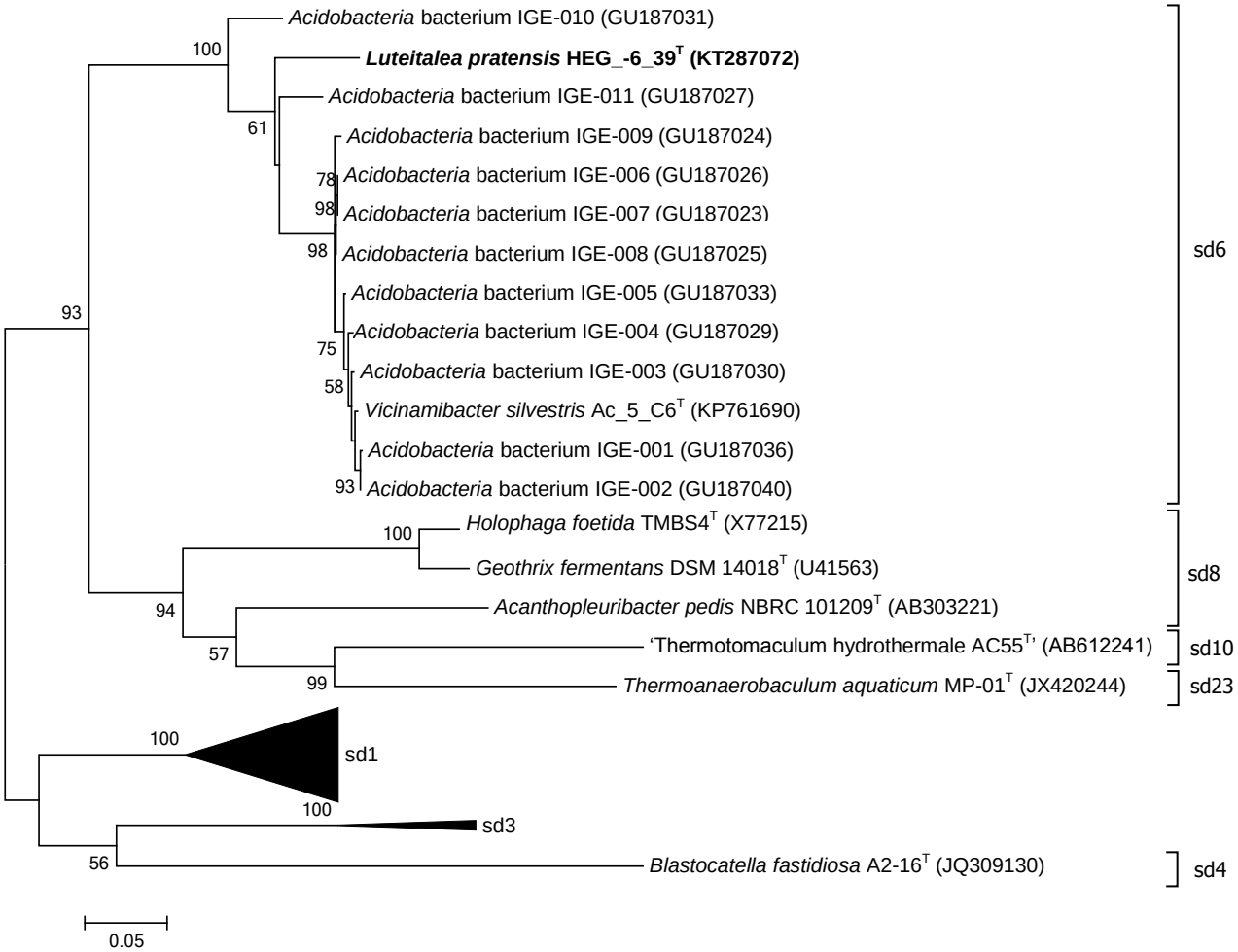


Figure 75 – Maximum likelihood (ML) phylogenetic tree based on almost full length 16S rRNA gene sequences, illustrating the phylogenetic position of *Luteitalea pratensis* HEG_-6_39^T. The best evolutionary model of nucleotide substitution applied was GTR+I+G. *Blastocatella fastidiosa* A2-16^T (JQ309130) was used as outgroup. Bar indicates 5% nucleotide divergence. Only bootstrap values above 50% are indicated at the branches (1000 replicates).

Strain HEG_-6_39^T differs from *Vicinamibacter silvestris* Ac_5_C6^T as it forms smaller cells capable of growth at significantly lower temperatures. The fatty acid profile between the two strains differs, being that HEG_-6_39^T possesses *iso*-$C_{15:0}$, summed feature 3 ($C_{16:1}$ *ω6c*/ $C_{16:1}$ *ω7c*) and $C_{18:1}$ *ω9c*, as major components, unlike its closest described relative where these fatty acids are residual or absent. Regarding the polar lipid composition, two unknown phospholipids are present and one glycolipid is absent, as compared with *Vicinamibacter silvestris* Ac_5_C6^T. Based on the phylogenetic, morphological, physiological, metabolic and chemotaxonomic properties of HEG_-6_39^T it was concluded that it represents a new genus and species within *Acidobacteria* sd6 for which the name *Luteitalea pratensis* gen. nov., sp. nov. was proposed.

6.1.8.1 – Description of *Luteitalea*, gen. nov.

Luteitalea (Lu.te.i.ta'le.a L. fem. adj. lutea, golden-yellow; L. fem. n. talea, a slender staff; a yellow pigmented, rod-shaped bacterium).

Gram-negative, non-motile, non-spore-forming, non-capsulated short rods that divide by binary fisson. Catalase positive and oxidase negative. Aerobic, chemoorganotrophic, mesophile. The respiratory quinone is MK-8. The major fatty acids are iso-C15:0, summed feature 3 (C16:1 ω6c/ C 16:1 ω7c), C 18:1 ω9c and iso-C17:1 ω9c Diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG) and two unknown phospholipids are the major polar lipids. The G+C content of genomic DNA is 64.7 mol%. The type species is *Luteitalea pratensis*.

6.1.8.2 – Description of *Luteitalea pratensis*, sp. nov.

Luteitalea pratensis (pra.ten'sis. L. fem. adj. pratensis growing in a meadow, referring to the isolation of the type strain from a grassland).

Cells are 0.9-1.4 µm long and 0.6-0.75 µm wide. Colonies are dark yellow, circular, convex with entire margins, reaching 0.5-1.0 mm in diameter. Grows at temperatures from 0-36°C (optimum at 20-30°C) and pH 5.3-8.3 (optimum at 6.1-7.5). Under optimal growth conditions doubling time is 29.8 h. The strain grows at NaCl concentrations up to 1%, but fastest at NaCl concentration of 0.25%.

Uses glucose, lactose, cellobiose, galactose, mannose, melezitose, raffinose, maltose, sucrose, trehalose, xylose, casamino acids, casein-hydrolysate, yeast extract, glycerin, peptone, gluconate, N-acetylgalactosamine Na-pyruvate, 2-oxogluconate, protocatechuate and aspartate as sole carbon source. No growth is observed on arabinose, fructose, fucose, sorbose, lyxose, rhamnose, lactate, adenitol, arabitol, mannitol, myo-inositol, sorbitol, xylitol, lysine, hydroxyproline, glycolate, malonate, propionate, oxaloacetate, lactate, butanol, ethanol, methanol, propanol, N-acetylglucosamine, caproate, caprylate, dulcitol, ethyleneglycol, erythrose, erythrulose, α-hydroxybutyrate, isocitrate, laevulinate, arabinose, glucosamine, glucoronate, lyxitol, crotonate, heptanoate, 2-oxoglutarate, acetoin, ascorbate, glyoxilate, 2-oxovalerate, maleic acid, 1,2-butandiol, 2,3-butandiol, 1,2-propandiol, alanine, leucine, arginine, asparagine, cysteine, glutamine, isoleucine, ornithine, proline, benzoate, tryptophan, acetate, butyrate, formiate, β-hydroxybutyrate, γ-hydroxybutyrate, isobutyrate, tyrosine, serine, phenylalanine, glycine, leucine, histidine, valine, methionine, threonine, nicotinic acid, tween 80, laminarin, adipate, succinate, shikimate, glutamate, malate, citrate, tartrate, fermented rumen extract, isovaleric acid, fumarate, heptanoate and trimethoxybenzoate.

Tests positive for alkaline and acid phosphatase, esterase C4, trypsin, α-chymotrypsin and naphtol-AS-BI-phosphohydrolase activities. Esterase lipase C8 and leucine arylamidase show weak activities. No activities of the enzymes lipase, valine arylamidase, cysteine arylamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Nitrate reduction to nitrite and/or nitrogen, indole production, fermentation of glucose, arginine dihydrolase, urease, gelatin and aesculin hydrolysis are negative. Resistance is observed against penicillin G, oxacillin, ampicillin, ticarcillin, mezlocillin, cefalotin, cefazolin, cefotaxime, aztreonam, imipenem, chloramphenicol, erythromycin, ofloxacin, norfloxacin, pipemidic acid, nitrofurantoin, neomycin, fosfomycin, moxifloxacin, linezolid and nystatin.

The type strain HEG_-6_39^T (= DSM 100886^T = KCTC 52215^T) was isolated from a mown pasture grassland soil in the Hainich-Dün region, Thuringia, Germany.

6.1.8.2 – Genome properties of *Luteitalea pratensis* HEG_-6_39^T

The genome contained 7,480,314 bp and 6,295 predicted protein coding genes (Huang *et al.* 2016) (Figure 76). The GC percentage was 64.7%. Only one rRNA operon was found. In addition, like other bacterial genomes, one tmRNA gene was found. The RAST annotation recognized one third of the CDSs as subsystem related. The most populated subsystem categories were carbohydrate (401), amino acids and derivatives (389) and RNA metabolism (184).

It is noteworthy that the genome contains 104 glycoside hydrolases and 183 peptidases. The complete gene sets for assimilatory nitrate reduction and sulfite reduction were found. Also identified are detoxification operons against arsenate, arsenite, antimonite, cobalt, zinc, lead, cadmium and mercury. Antimicrobial resistance genes, such as mdtABC drug exporter genes, were also found. The HEG_-6_39 genome encodes three cold-shock protein genes cspADE and two pathways for trehalose biosynthesis.

The nucleotide sequence has been deposited at GenBank under the accession number CP015136.

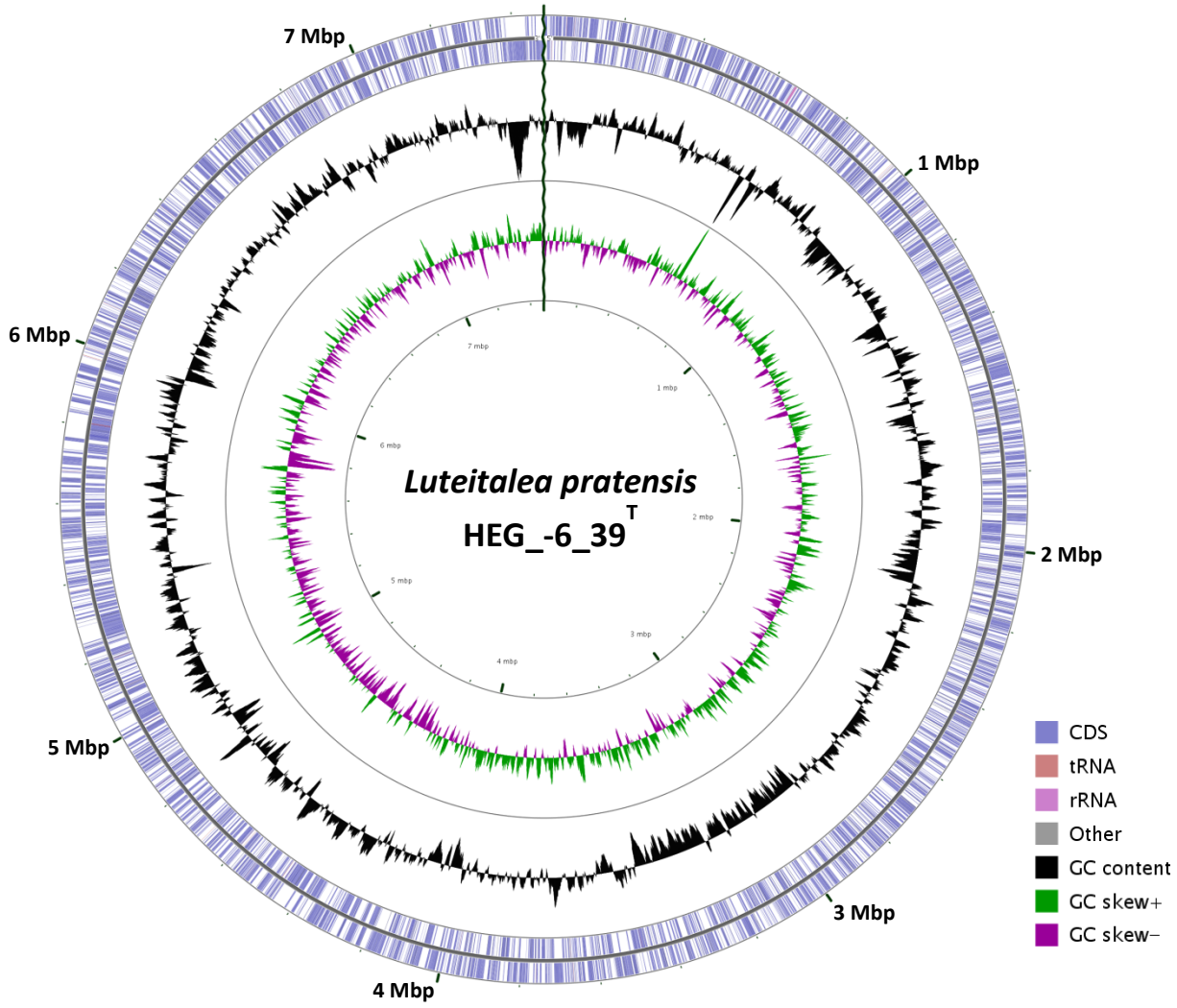


Figure 76 – Genomic representation of *Luteitalea pratensis* HEG_-6_39T genome, with CGView Server (Grant & Stothard 2008).

6.2 – Discussion

6.2.1 – Low nutrient solid media are suitable for cultivating novel oligotrophic soil bacteria

It is well known that bacterial cultivation efforts so far are biased towards certain taxonomic groups. Even amongst the taxonomic groups which possess cultivated representatives, the majority are still understudied since most of the isolated bacteria belong to only 4 phyla (Overmann *et al.* 2017). Not only is cultivation biased towards certain phyla it is also biased towards fast growing bacteria. With the use of long incubation times and the use of oligotrophic media it was possible to isolate from natural temperate grassland and forest soils several members of rarely isolated groups.

An influence of the sample was observed irrespectively of the medium and approach used, with culturability values and number of isolated bacteria being generally lower in samples from forest soil when compared to their grassland counterpart (soil from the same geographic region). This is likely related with pH, since the one used throughout the experiments was neutral (pH 7), which is closer to the pH values registered the grassland samples (5.74 – 7.05) as opposed to forest samples (3.6 – 6.15). This can be additionally explained by the distinct bacterial community structures present in the distinct samples (Figure 47), which determine bacterial interactions (Stubbendieck *et al.* 2016).

The three different approaches used in this study were selected in order to target different bacteria with specific requirements. The high throughput liquid media dilution approach was selected with the aim of enriching sensitive bacteria that do not grow in solid media and which may be affected by antibacterial substances produced by the neighbouring bacteria. In some cases high culturability values were obtained (up to 32.2%), which validates the use of these media for the cultivation of soil bacteria. These results need to be taken with caution, since it was clear from the well screening that more than 5 distinct bacteria were growing in many of the individual wells (maximum theoretical inoculated cells were 5). This is due to probability but also due to the introduction of soil particles with associated bacteria, since the majority of soil microorganisms lives in close contact with surfaces (Mills 2003). In other studies values of culturability were generally lower. In the case of soil from Ellinbank, the mean viable count with liquid DNB medium was only 1.4% of the mean microscopically determined total cell count (Janssen *et al.* 2002). Recently this method was employed to retrieve novel members of underrepresented groups which are ubiquitous in soils, such as the first member of *Acidobacteria* subgroup 6, *Vicinamibacter silvestris* (Huber *et al.* 2016) and members of *Rubrobacteria*, a deep branching class of *Actinobacteria* (Foesel *et al.* 2016b). Although all media employed in the high throughput liquid media dilution approach featured low nutrient concentrations, they nevertheless targeted distinct oligothrophic bacteria. The medium with a mixture of polysaccharides, SSE/Polymermix, enriched bacteria affiliated with members of

Bacillales and *Micrococcales*, orders with members known for polysaccharide degradation (Taylor *et al.* 2012; Chakdar *et al.* 2016). Surprisingly, the known quintessential degraders of polysaccharides in soil, the members of *Actinomycetales* (Yeager *et al.* 2017), were not very abundant in this medium. Both BEX and SSE were supplemented with HD1:10, but despite the nutrient similarity, both enrichments and isolated strains were distinct between the two media. The medium with low amounts of sugars, fatty acids, aminoacids, TCA intermediaries and aromatic compounds, SSE/Cmix, yielded the best results in terms of amount and novelty of the isolated bacteria, allowing the isolation of novel members of the underrepresented groups of *Acidobacteria* and *Rubrobacteria*. The analysis of the interesting bimodal pattern observed for the enrichments of SSE/Cmix and SSE/Polymermix media suggest the existence of two community types characterized by either low or high taxon richness, which existence could be linked to bacterial interactions. Multiple partners are likely important in the enrichments with a high taxon richness, or keystone species are present (Berry & Widder 2014) which trigger the development of more complex communities. On the other hand, antagonist bacteria may be present in the enrichments with low taxon richness, which may impede the development of more complex communities.

The cultivation approach targeting bacteria adhering and multiplying on solid surfaces, despite allowing the isolation of known biofilm forming bacteria, such as *Pseudomonas* and *Bacillus*, was the least successful method for obtaining novel isolates from soil. Contrary to another study with the same surfaces which retrieved 12 novel phylotypes from a soil sample (Gich *et al.* 2012), all the isolates recovered in this study were identical to previously cultured bacteria. Nevertheless, it was clear that this approach targeted bacteria which the other approaches could not. Moreover, the species sample coverage was low for this approach, which reveals that not all potential is exhausted. This may be further perfected in the light of novel insights into biofilm development in soil pores, which reveal that cells in slow growing biofilms can have a competitive advantage by allowing flow and dispersal of nutrients, while fast growing biofilms can choke of their own nutrient supply (Coyte *et al.* 2017).

Previous cultivation trials with soil bacteria have indicated an improved cultivation success using solidified media (Janssen *et al.* 2002; Schoenborn *et al.* 2004; Senechkin *et al.* 2010; Pulschen *et al.* 2017). This is in agreement with this study, since more diverse and novel organisms could be obtained with direct plating when compared to the other approaches used. The comparison of these results with the ones from the high throughput liquid media dilution and biofilm targeting approaches needs to be made with caution, since the SSE/HP medium is only a less rich version of the SSE/HD1:10, which could explain the different outputs. The conditions selected were verified to target oligotrophic bacteria, since most of the colonies developed during a large period and most of

the isolated bacteria exhibits fastidious growth. Culturability values were generally lower than other studies (Janssen *et al.* 2002; da Rocha *et al.* 2010), but this may relate to the fact that microcolonies were not taken into account (Davis *et al.* 2011). Nevertheless, a high proportion (around one third) of all selected colonies were novel, and this holds true for the distinct soil environments tested (grassland and forest soils versus swamp soil). The good output and reproducibility observed makes these conditions not only the best tested, but suitable to assist in uncovering the potential of not-yet cultivated oligotrophic soil bacteria.

Cultivation efforts in recent years keep revealing novel bacteria at a steady pace, but the majority of these represent novel species of already known genera (Overmann *et al.* 2017). This precludes that novel traits and functions are investigated since is likely that phylogenetically close individuals have similar characteristics and inhabit similar niches. With this study, the majority of isolated strains represents novel genera, such as *Luteitalea pratensis* HEG_-6_39^{T}. Some even represent first representatives of higher taxonomic ranks, such as HEG_-6_68, Swamp8 and Swamp150 which likely represent a class within *Chloroflexi*. This phyla is known for harbouring very diverse organisms with distinct metabolic lifestyles, including photoautotrophs (Gaisin *et al.* 2017), fermentative bacteria (Yamada *et al.* 2006), organohalide respiring organisms (Löffler *et al.* 2013) and aerobic thermophiles (Jackson *et al.* 1973). Novel lineages likely harbour novel metabolic features which may not only extend our knowledge of such organisms, but also be exploited for biotechnological applications.

6.2.2 – Bacterial co-occurrence as basis for co-cultivation

Coupled with high throughput liquid media dilution approach, the screening of the inventory growing in the individual wells through sequencing of the V1-V2 region of the 16S rRNA gene, was performed, which allowed the selection of target enrichments for plating. This greatly improves cultivation efforts and allows high throughput, since it reduces time and resources employed in plating a multitude of non-target enrichments and it could be used in the future as a means to increase plating efficiency, since it allows for plating different target organisms in selective conditions. The screening of the inventory growing in the individual wells can also provide useful insights into bacterial interactions, by detecting pairs of organisms which co-occur more frequently than expected by random chance. Bacteria are social organisms which interact with other species while responding to their environments (West *et al.* 2007). The analysis of co-occurrence data has long been used by ecologists to disentangle the forces that rule community structure, but this has hardly been applied to cultivation efforts.

The putative negative interactions observed in this study can be explained by production of antagonistic molecules, such as antibiotics. For the bacterial genus which establish the largest amount of negative relationships, a member, *Georgenia ruanni* was isolated during a screening programme for new antibiotics, and it was confirmed that it contained both type I and type II polyketide biosynthesis pathway genes (Li *et al.* 2007). Other important taxa, such as members of *Bacilli* are known for the production of antibiotics, especially polypeptides (Yilmaz *et al.* 2006; Malanicheva *et al.* 2014).

Positive relationships can be established in various ways, from cooperation to build biofilms which confers antibiotic resistance to its members (Rodrıguez-Martinez *et al.* 2006) to syntrophic associations where two or more species exchange metabolic products with mutual benefits (Morris *et al.* 2013). Putative positive interactions were detected, where members of *Bacilli* had a prominent role. Syntrophic relationships have been detected for members of this group in an artificial symbiosis experiment, with *Azotobacter vinelandii* fixing nitrogen, *Bacillus licheniformis* degrading penicillin, and *Paenibacillus curdlanolyticus* breaking down cellulose into a usable carbon source (Kim *et al.* 2008). All capacities were required for viability of the strains in defined medium. Despite this, non-random co-occurrences may also stem from the co-localization of bacteria in the same soil particles, which doesn't represent an interaction, but the occupation of the same niche.

6.2.3 – Novel isolates are important in different soil compartments

When evaluating the importance of the novel isolates in the environments which are the focus of this thesis, namely the rhizosphere of grassland plants (Chapter 4) and the colonization of soil minerals (Chapter 5), not many are found in high abundance. Interestingly, strain 88_S_G_1, which likely represents a novel species within the genus *Sphingosinicella*, is the most active novel isolate in both rhizosphere and newly introduced soil minerals. *Alphaproteobacteria* are generally enriched and active in plant rhizospheres, and members of this genus have been reported as associated with the rhizosphere of maize (van Wyk *et al.* n.d.), soybean (Liang *et al.* 2014) and cucumber (Akter *et al.* 2015). It is unexpected to find this bacterium as associated with minerals, although *Sphingosinicella* members have been found as inhabitants of an high Arctic polar desert rocks (Choe *et al.* 2018). In fact, with the increase registered over time, it is more likely that this bacterium is a secondary colonizer, utilizing resources released by others and leachates from the surrounding soil.

Although most of the novel isolates were rare in the environments analysed, many may be important in others. One example is the selected strain for characterization, HEG_-6_39^T, which is abundant in other soil environments. This strain has been detected to be enriched in cold desert

valleys of Antartica, and HEG_-6_39^T is indeed capable of growth at 0°C. Moreover, genes for cold shock proteins and trehalose biosynthesis are found in the genome, all of which denote an adaptation to cold environments. The psychrotolerance observed for strain HEG_-6_39^T is a trait shared with other acidobacterial genera, namely *Granulicella*, *Bryobacter* and *Paludibaculum*, all isolated from Northern Russia peat bogs or Northern Finland soils (Kulichevskaya *et al.* 2010, 2014; Pankratov & Dedysh 2010). It is also important to consider that rare bacteria can influence community structure and play essential roles in community functioning and stability (Jia *et al.* 2018). They serve as a reservoir of species and functional traits, which may become important under changing conditions (Jousset *et al.* 2017). Moreover, they can have a large functional effect in ecosystems, as was shown for *Desulfosporosinus*. This bacterium was the most important sulfate reducer in a peatland despite making up only 0.006% of the total community (Pester *et al.* 2010).

6.2.4 – Summarizing conclusion

Various novel information could be gained from this work, which can assist in improving cultivation of novel bacterial lineages. Although valuable information on bacterial interactions could be derived from the liquid media cultivation, the best approach for obtaining novel soil bacterial isolates would be direct plating. This method yielded many novel strains, many from understudied groups, an accomplishment not commonly obtained from a single approach from other cultivation efforts. Moreover, these conditions enable a good output in various soil environments. As observed, the potential of this approach for the isolation of novel bacteria is far from exhausted, and the extension of this to other soil environments coupled with modifications to the basal media SSE/HP (different pH, different incubation temperature, addition of unusual carbon sources, etc) should allow the retrieval of a vast array of novel soil bacteria from various lineages.

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6.4 – Supplementary information

Supplementary Table 5 – Closest validly described species to the novel strains isolated from grassland and forest soils with the high throughput liquid media dilution cultivation, obtained used the EzBiocloud identification tool (Yoon *et al.* 2017).

Isolate	Closest relative (EzBioCloud)	Similarity	Phylum	Class	Order	Family
0163_2	*Angustibacter aerolatus* 7402J-48(T)	96.53%	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Kineosporiaceae*
095_11	*Stenotrophobacter roseus* Ac_15_C4(T)	96.26%	*Acidobacteria*	*Blastocatellia*	*Blastocatellales*	*Blastocatellaceae*
0141_2	*Conexibacter arvalis* KV-962(T)	95.10%	*Actinobacteria*	*Actinobacteria*	*Solirubrobacterales*	*Conexibacteraceae*
0166_1	*Conexibacter arvalis* KV-962(T)	95.46%	*Actinobacteria*	*Actinobacteria*	*Solirubrobacterales*	*Conexibacteraceae*
078_4	*Dongia mobilis* CGMCC 1.7660(T)	95.20%	*Proteobacteria*	*Alphaproteobacteria*	*Rhodospirillales*	*Rhodospirillaceae*
275_S_G_1	*Flavitalea populi* HY-50R(T)	95.22%	*Bacteroidetes*	*Sphingobacteriia*	*Sphingobacteriales*	*Chitinophagaceae*
60_S_A_2	*Flavobacterium aquatile* LMG 4008(T)	96.68%	*Bacteroidetes*	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*
0127_4	*Hyphomonas neptunium* ATCC 15444(T)	89.18%	*Proteobacteria*	*Alphaproteobacteria*	*Caulobacterales*	*Hyphomonadaceae*
150_B_G_1	*Labilithrix luteola* DSM 27648(T)	95. 87%	*Proteobacteria*	*Deltaproteobacteria*	*Mycrococcales*	*Labilitrichaceae*
244_B_G_1	*Microvirga aerophila* 5420S-12(T)	96.59%	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Methylobacteriaceae*
0125_3	*Nitrosospira multiformis* ATCC 25196(T)	90.60%	*Proteobacteria*	*Betaproteobacteria*	*Nitrosomonadales*	*Nitrosomonadaceae*
0111_1	*Oceanibaculum pacificum* MCCC 1A02656(T)	92.40%	*Proteobacteria*	*Alphaproteobacteria*	*Rhodospirillales*	*Rhodospirillaceae*
078_1	*Erythrobacter aquimixticola* JSSK-14(T)	95.52%	*Proteobacteria*	*Alphaproteobacteria*	*Sphingomonadales*	*Erythrobacteraceae*
88_S_G_1	*Sphingosinicella vermicomposti* YC7378(T)	95.96%	*Proteobacteria*	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*
31_S_G_5	*Terrimonas lutea* DY(T)	91.61%	*Bacteroidetes*	*Sphingobacteriia*	*Sphingobacteriales*	*Chitinophagaceae*
7_S_G_1	*Terrimonas pekingensis* QH(T)	95.66%	*Bacteroidetes*	*Sphingobacteriia*	*Sphingobacteriales*	*Chitinophagaceae*
0208_1	*Undibacterium jejuense* JS4-4(T)	91.67%	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Oxalobactereaceae*

Supplementary Table 6 – Closest validly described species to the novel strains isolated from grassland and forest soils with the direct plating cultivation approach, obtained used the EzBiocloud identification tool (Yoon *et al.* 2017).

Isolate	Closest relative (EzBioCloud)	Similarity	Phylum	Class	Order	Family
SEG_-6_44	*Aciditerrimonas ferrireducens* IC-180(T)	93.06%	*Actinobacteria*	*Actinobacteria*	*Acidimicrobiales*	
AEW_-6_39	*Afipia birgiae* 34632(T)	94.68%	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Rhodobiaceae*
HEG_-6_68	*Anaerolinea thermophila* UNI-1(T)	83.39%	*Chloroflexi*	*Anaerolinae*	*Anaerolinales*	
AEW_-6_31	*Angustibacter aerolatus* 7402J-48(T)	96.65%	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Kineosporiaceae*
AEW_-7_5	*Aquihabitans daechungensis* CH22-21(T)	94.57%	*Actinobacteria*	*Actinobacteria*	*Acidimicrobiales*	*Iamiaceae*
SEG_-6_13	*Armatimonas rosea* YO-36(T)	92.84%	*Armatimonadetes*	*Armatimonadia*	*Armatimonadales*	*Armatimonadaceae*
HEG_-6_71	*Bauldia consociata* 11(T)	94.73%	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	
AEG_-7_2	*Conexibacter woesei* DSM 14684(T)	92%	*Actinobacteria*	*Actinobacteria*	*Solirubrobacterales*	*Conexibacteriaceae*
HEG_-6_24	*Conexibacter woesei* DSM 14684(T)	91.34%	*Actinobacteria*	*Actinobacteria*	*Solirubrobacterales*	*Conexibacteriaceae*
HEG_-6_1	*Denitratisoma oestradiolicum* AcBE2-1(T)	91.31%	*Proteobacteria*	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*
SEG_-6_39	*Dokdonella kunshanensis* DC-3(T)	94.93%	*Proteobacteria*	*Gammaproteobacteria*	*Lysobacterales*	*Rhodobaceraceae*
AEG_-6_29	*Dongia mobilis* LM22(T)	95.23%	*Proteobacteria*	*Alphaproteobacteria*	*Rhodospirillales*	*Rhodospirillaceae*
HEW_-6_17	*Flavitalea populi* HY-50R(T)	95.02%	*Bacteroidetes*	*Shpingobacteriia*	*Sphingobacteriales*	*Chitinophagaceae*
AEW_-6_25	*Flavobacterium hercynium* WB 4.2-33(T)	96.19%	*Bacteroidetes*	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*
HEG_-6_33	*Haliangium ochraceum* DSM 14365(T)	89.73%	*Proteobacteria*	*Deltaproteobacteria*	*Myxococcales*	*Haliangiaceae*
AEG_-7_6	*Hyphomicrobium sulfonivorans* S1(T)	96.57%	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*
HEG_-6_27	*Ilumatobacter fluminis* YM22-133(T)	96.04%	*Actinobacteria*	*Actinobacteria*	*Acidimicrobiales*	*Acidimicrobiaceae*
SEG_-6_41	*Ilumatobacter fluminis* YM22-133(T)	93.70%	*Actinobacteria*	*Actinobacteria*	*Acidimicrobiales*	*Acidimicrobiaceae*
AEW_-6_42	*Lysobacter dokdonensis* DS-58(T)	95.91%	*Proteobacteria*	*Gammaproteobacteria*	*Lysobacterales*	*Lysobactereaceae*
AEW_-6_47	*Micromonospora auratinigra* TT1-11(T)	96.53%	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Micromonosporaceae*
HEG_-6_10	*Oceanibaculum pacificum* MC2UP-L3(T)	89.43%	*Proteobacteria*	*Alphaproteobacteria*	*Rhodospirillales*	*Rhodospirillaceae*
SEG_-6_22	*Opitutus terrae* PB90-1(T)	93.42%	*Verrucomicrobia*	*Opitutae*	*Opitutales*	*Opitutaceae*

HEG_-6_31	*Patulibacter ginsengiterrae* P4-5(T)	91.18%	*Actinobacteria*	*Actinobacteria*	*Solirubrobacterales*	*Patulibactereaceae*
AEW_-7_8	*Patulibacter medicamentivorans* I11(T)	91.46%	*Actinobacteria*	*Actinobacteria*	*Solirubrobacterales*	*Patulibactereaceae*
AEW_-6_35	*Pirellula staleyi* DSM 6068(T)	91.58%	*Planctomycetes*	*Planctomycea*	*Planctomycetales*	*Planctomycetaceae*
HEG_-6_57	*Rhizomicrobium electricum* Mfc52(T)	93.62%	*Proteobacteria*	*Alphaproteobacteria*		
AEW_-7_2	*Rudaea cellulosilytica* DSM 22992(T)	95.30%	*Proteobacteria*	*Gammaproteobacteria*	*Lysobacterales*	*Rhodobactereaceae*
HEW_-8_3	*Sphaerobacter thermophilus* DSM 20745(T)	88.29%	*Chloroflexi*	*Thermomicrobia*	*Sphaerobacterales*	*Sphaerobacteraceae*
HEG_-6_52	*Sporichthya polymorpha* DSM 43042(T)	95.10%	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Sporichtyaceae*
SEG_-6_5	*Sporichthya polymorpha* DSM 43042(T)	94.85%	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Sporichtyaceae*
SEG_-6_3	*Terrimonas lutea* DY(T)	91.80%	*Bacteroidetes*	*Shpingobacteriia*	*Sphingobacteriales*	*Chitinophagaceae*
HEG_-6_56	*Terrimonas pekingensis* QH(T)	94.03%	*Bacteroidetes*	*Shpingobacteriia*	*Sphingobacteriales*	*Chitinophagaceae*
AEW_-6_42	*Lysobacter dokdonensis* DS-58(T)	95.91%	*Proteobacteria*	*Gammaproteobacteria*	*Lysobacterales*	*Lysobactereaceae*
HEG_-6_21	*Variibacter gotjawalensis* GJW-30(T)	95.38%	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	
HEG_-6_39	*Vicinamibacter silvestris* Ac_5_C6(T)	93.56%	*Acidobacteria*			

Supplementary Table 7 – Closest validly described species to the novel strains isolated from the swamp sample with the direct plating cultivation approach, obtained used the EzBiocloud identification tool (Yoon *et al.* 2017).

Isolate	**Closest relative (EzBioCloud)**	**Similarit**	**Phylum**	**Class**	**Order**	**Family**
Swamp95	*Aciditerrimonas ferrireducens* IC-180(T)	92.66%	*Actinobacteria*	*Actinobacteria*	*Acidimicrobiales*	
Swamp8	*Anaerolinea thermophila* UNI-1(T)	82.75%	*Chloroflexi*	*Anaerolinae*	*Anaerolinales*	
Swamp103	*Aquihabitans daechungensis* CH22-21(T)	93.46%	*Actinobacteria*	*Actinobacteria*	*Acidimicrobiales*	*Iamiaceae*
Swamp134	*Sulfuritalea hydrogenivorans* DSM 22779(T)	91.40%	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*
Swamp35	*Chryseolinea serpens* DSM 24574(T)	95.31%	*Bacteroidetes*	*Cytophagia*	*Cytophagales*	
Swamp40	*Chryseolinea serpens* DSM 24574(T)	93.85%	*Bacteroidetes*	*Cytophagia*	*Cytophagales*	

Swamp169	*Crocinitomix catalasitica* IFO 15977(T)	85.98%	*Bacteroidetes*	*Flavobacteriia*	*Flavobacteriales*	
Swamp33	*Denitratisoma oestradiolicum* AcBE2-1(T)	92.31%	*Proteobacteria*	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*
Swamp159	*Denitratisoma oestradiolicum* AcBE2-1(T)	95.75%	*Proteobacteria*	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*
Swamp49	*Dongia mobilis* CGMCC 1.7660(T)	95.52%	*Proteobacteria*	*Alphaproteobacteria*	*Rhodospirillales*	*Rhodospirillaceae*
Swamp77	*Gemmatimonas aurantiaca* T-27(T)	95.43%	*Gemmatimonadetes*	*Gemmatimonadetes*	*Gemmatimonadales*	*Gemmatimonadaceae*
Swamp109	*Gemmatimonas aurantiaca* T-27(T)	86.14%	*Gemmatimonadetes*	*Gemmatimonadetes*	*Gemmatimonadales*	
Swamp164	*Methyloversatilis universalis* FAM5(T)	90.51%	*Proteobacteria*	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*
Swamp113	*Nitrosospira multiformis* ATCC 25196(T)	90.46%	*Proteobacteria*	*Betaproteobacteria*	*Nitrosomonadales*	*Nitrosomonadaceae*
Swamp67	*Nitrosospira multiformis* ATCC 25196(T)	90.73%	*Proteobacteria*	*Betaproteobacteria*	*Nitrosomonadales*	*Nitrosomonadaceae*
Swamp84	*Nocardioides panacihumi* Gsoil 616(T)	96.02%	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Nocardioidaceae*
Swamp73	*Ohtaekwangia koreensis* DSM 25262(T)	92.03%	*Bacteroidetes*	*Cytophagia*	*Cytophagales*	
Swamp93	*Opitutus terrae* PB90-1(T)	95.14%	*Verrucomicrobia*	*Opitutae*	*Opitutales*	*Opitutaceae*
Swamp116	*Opitutus terrae* PB90-1(T)	93.58%	*Verrucomicrobia*	*Opitutae*	*Opitutales*	*Opitutaceae*
Swamp150	*Ornatilinea apprima* P3M-1(T)	83.53%	*Chloroflexi*	*Anaerolinae*	*Anaerolinales*	
Swamp196	*Arcticibacter pallidicorallinus* Hh36(T)	83.59%	*Bacteroidetes*	*Sphingobacteriia*	*Sphingobacteriales*	*Sphingobacteriaceae*
Swamp70	*Pseudolabrys taiwanensis* CC-BB4(T)	94.53%	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Xanthobactereaceae*
Swamp83	*Pseudorhodoplanes sinuspersici* RIPI 110(T)	95.24%	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*
Swamp60	*Sporichthya polymorpha* DSM 43042(T)	94.31%	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Sporichtyaceae*
Swamp72	*Sporichthya polymorpha* DSM 43042(T)	94.55%	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Sporichtyaceae*
Swamp38	*Terrimonas arctica* R9-86(T)	96.59%	*Bacteroidetes*	*Shpingobacteriia*	*Sphingobacteriales*	*Chitinophagaceae*
Swamp108	*Pseudorhodoplanes sinuspersici* RIPI 110(T)	95.32%	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*
Swamp99	*Wohlfahrtiimonas larvae* KBL006(T)	93.49%	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonadales*	*Xanthomonadaceae*
Swamp78	*Youhaiella tibetensis* F4(T)	97%	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*

Supplementary Table 7 - Carbon source utilization by HEG_-6_39^T (1) when compared with the closest relative *Vicinamibacter silvestris* Ac_5_C6^T (2), +, positive; -, negative; (+), weak growth detected.

Substrates	1	2	Substrates	1	2	Substrates	1	2
Arabinose	-	+	Histidine	-	-	Glyoxylate	-	-
Cellobiose	+	+	Hydroxy-Proline	-	+	Heptanoate	-	(+)
Erythrose	-	-	Isoleucine	-	-	Isovalerian acid	-	(+)
Erythrulose	-	-	Leucine	-	-	Laevulinate	-	-
Fructose	-	+	Lysine	-	-	Lactate	-	-
Fucose	-	(+)	Methionine	-	-	Malate	-	-
Galactose	+	+	Ornithine	-	-	Maleic acid	-	(+)
Glucose	+	+	Lysine	-	-	Malonate	-	-
Lactose	+	+	Methionine	-	-	Nicotinic acid	-	-
Lyxose	-	+	Ornithine	-	-	Oxaloacetate	-	-
Maltose	+	+	Phenylalanine	-	-	Propionate	-	-
Mannose	+	(+)	Proline	-	(+)	Protocatechuate	+	(+)
Melezitose	+	+	Serine	-	-	Pyruvate	+	(+)
Raffinose	+	+	Threonine	-	-	Shikimate	-	(+)
Rhamnose	-	+	Tryptophan	-	-	Succinate	-	-
Sorbose	-	-	Tyrosine	-	-	Tartrate	-	-
Sucrose	+	+	Valine	-	-	2-Oxovalerate	-	-
Trehalose	+	+	Adipate	-	-	Butanol	-	-
Xylose	+	+	Acetate	-	-	1,2-Butandiol	-	-
Gucosamine	-	-	Ascorbate	-	(+)	2,3-Butandiol	-	-
N-acetylglucosamine	+	+	Benzoate	-	-	Ethanol	-	-
N-acetylgalactosamine	-	+	Trimethoxybenzoate	-	(+)	Ethylene glycol	-	-
Acetoin	-	(+)	Butyrate	-	-	Glycerol	+	(+)
Adonitol	-	-	α-hydroxybutyrate	-	-	Methanol	-	-
Arabitol	-	-	β- Hydroxybutyrate	-	-	Propanol	-	-
Dulcitol	-	-	γ-Hydroxybutyrate	-	-	1,2-Propandiol	-	-
Lyxitol	-	-	Isobutyrate	-	-	Fermented rumen extract	-	+
Mannitol	-	-	Caproate	-	-	Tween 80	-	-
Myo-Inositol	-	-	Caprylate	-	-	Casamino acids	+	+
Sorbitol	-	-	Citrate	-	(+)	Casein hydrolysate	+	+
Xylitol	-	-	Isocitrate	-	-	Peptone	+	+
Alanine	-	(+)	Crotonate	-	-	Yeast extract	+	+
Arginine	-	-	Formate	-	-	Lamiarin	-	(+)
Asparagine	-	(+)	Fumarate	-	+	Cellulose	-	-
Aspartate	+	(+)	Gluconate	+	+	Chitin	-	-
Cysteine	-	-	2-Oxogluconate	+	-	Starch	-	-
Glutamate	-	+	Glucuronate	-	-	Pectin	-	-
Glutamine	-	-	2-Oxoglutarate	-	(+)	Xylan	-	-
Glycine	-	-	Glycolate	-	-			

Supplementary Table 9 - Polar lipid and fatty acid profile of HEG_-6_39^T compared with the type strains of the closest *Acidobacteria* genera. Strains:1, HEG_-6_39^T; 2, *Vicinamibacter silvestris* Ac_5_C6^T,3, *Acanthopleuribacter pedis* FYK2218^T; 4, *Geothrix fermentans* H-5^T(Coates *et al.* 1999); 5, *Holophaga foetida* TMBS4^T(Liesack *et al.* 1994); 6, 'Thermotomaculum hydrothermale' AC55^T; 7, *Thermoanaerobaculum aquaticum* MP-01^T;

-, negative; ND, no data available; MC, major component; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol; uP, unidentified phospholipid; uG, unidentified glycolipid.

For fatty acids, only values above 1% are represented.

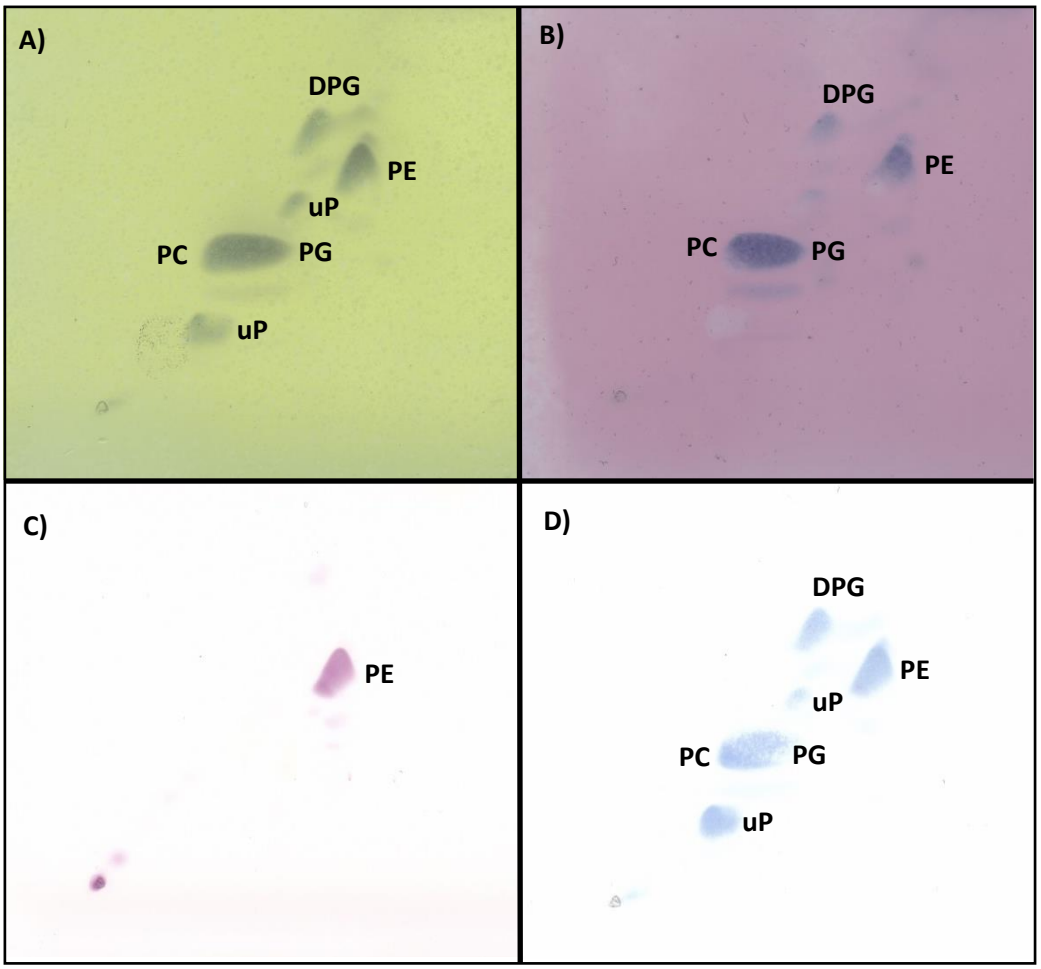
	1	2	3	4	5	6	7
Polar lipids	DPG, PE, PG, PC, uP	DPG, PE, PG, PC, uG	ND	ND	ND	PE, uP	DPG, PE, PG, uP
Major fatty acids (%)							
iso-$C_{11:0}$	1.4	-	-	ND	ND	-	-
iso-$C_{13:0}$	1.2	-	6.7	ND	ND	-	-
iso-$C_{13:0}$ 3-OH	1.7	1.8	5.7	ND	ND	-	-
iso-$C_{15:0}$	30.3	13.4	19.0	ND	ND	MC	28.3
$C_{16:0}$	2.2	4.4	12.5	ND	ND	-	5.3
iso-$C_{16:0}$	-	-	-	ND	ND	-	12.7
$C_{16:1}$ *ω6c*/ $C_{16:1}$ *ω7c*	18.4	5.6	-	ND	ND	-	-
iso-$C_{17:0}$	4.95	5.4	14.1	ND	ND	MC	23.7
anteiso-$C_{17:0}$	-	-	-	ND	ND	-	11.0
iso-$C_{17:1}$ *ω9c*	15.7[a]	30.1[a]	-	ND	ND	-	-
$C_{18:1}$	2.8	-	-	ND	ND	-	-
iso-$C_{18:0}$	-	-	-	ND	ND	-	9.1
$C_{18:1}$ *ω7c*	1	28.6	-	ND	ND	-	-
$C_{18:1}$ *ω9c*	17.1	-	-	ND	ND	-	-
cyclo-$C_{19:0}$ ω8c	-	1.4	-	ND	ND	-	-

[a] Determined as *iso*-C17:1 *ω9c* by the DSMZ service unit according to the MIDI System, but might be identified as *iso*-C17:1 *ω7c* by GC/MS as described previously(Foesel *et al.* 2016a).

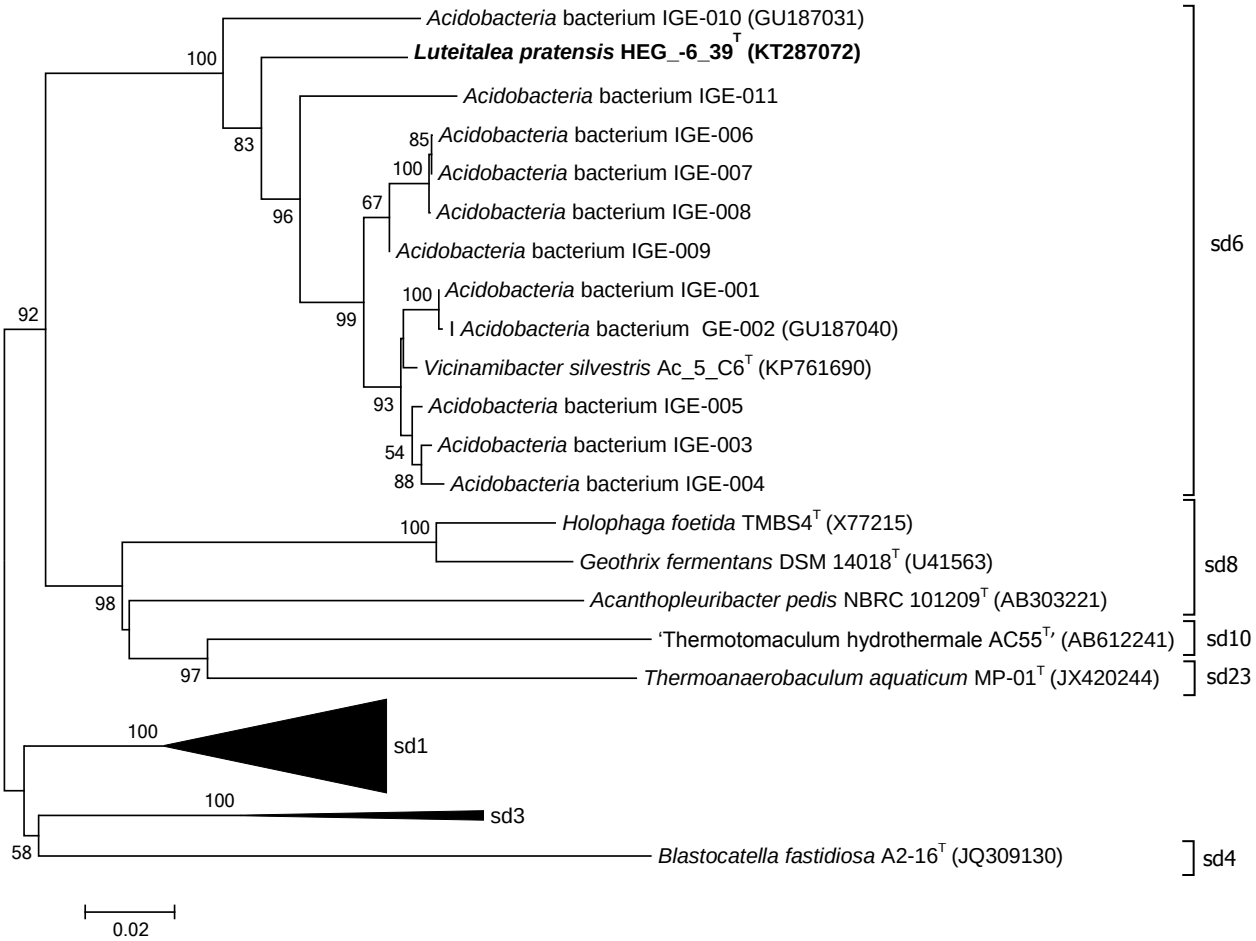
Supplementary Table 10. Antibiotic resistance profile of HEG_-6_39^T (1) when compared with the closest relative *Vicinamibacter silvestris* Ac_5_C6^T (2); ND, no data available; S, sensitive; R, resistant.

Antibiotics	**1**	**2**	Antibiotics	**1**	**2**
Amikacin	S	ND	Mezlocillin	R	ND
Ampicillin	R	R	Moxifloxacin	R	ND
Aztreonam	R	ND	Neomycin	R	ND
Bacitracin	S	ND	Nitrofurantoin	R	ND
Carbanicillin	ND	R	Norfloxacin	R	ND
Cefalotin	R	ND	Novobiocin	ND	ND
Cefazolin	R	ND	Nystatin	R	ND
Cefotaxime	R	ND	Ofloxacin	R	ND
Ceftriaxone	S	ND	Oxacillin	R	ND
Chloramphenicol	R	R	Penicillin G	R	ND
Clindamycin	S	ND	Pipemidic acid	R	ND
Colistin	S	ND	Piperacillin/tazobactam	S	ND
Doxycyclin	S	ND	Polymyxin B	S	ND
Erythromycin	R	R	Quinupristin/Dalfopristin	S	ND
Fosfomycin	R	ND	Spectinomycin	ND	S
Gentamicin	S	R	Streptomycin	ND	S
Imipenem	R	ND	Teicoplanin	S	ND
Kanamycin	S	R	Tetracycline	S	R
Lincomycin	S	ND	Ticarcillin	R	ND
Linezolid	R	ND	Vancomycin	S	ND

Supplementary Figure 2 - Polar lipid composition of strain HEG_-6_39^T on thin layer chromatography. Staining for determination of the polar lipids with dodecamolydophosphoric acid (A), anisaldehyde sulfuric acid (B), ninhydrin (C), molybdenum blue (D). For lipid separation chloroform:methanol:water (65:25:4, v/v/v) was used in the first direction and chloroform:methanol:acetic acid:water (80:12:15:4, v/v/v/v) in the second direction. PE: phosphatidylethanolamine, PC: phosphatidylcholine, DPG: diphosphatidylglycerol, PG: phosphatidylglycerol, uP: unidentified phospholipid.



Supplementary Figure 3 - Neighbor-joining (NJ) phylogenetic tree based on almost full length 16S rRNA gene sequences illustrating the phylogenetic position of *Luteitalea pratensis* HEG_-6_39^T. The evolutionary distances were computed using the Kimura 2-parameter model and are in the units of the number of base substitutions. *Blastocatella fastidiosa* A2-16^T (JQ309130) was used as outgroup. Bar indicates 2% nucleotide divergence. Only bootstrap values above 50% are indicated at the branches (1000 replicates).



Chapter 7 – Conclusion

The information on the functional role of individual bacteria in soils is restricted to only a few species and even less is known about how particular species interacts with other members of the microbial community. Evidence on how bacterial communities are established and develop over time in soil environments and niches is also rare. This study contributes to the deeper understanding of the colonization of the rhizosphere and of nutrient loaded minerals introduced in natural soil environments (temperate grassland ecosystems).

Employing cultivation independent techniques this study identifies the drivers which mediate bacterial community establishment in grassland plant rhizospheres. An overwhelming effect of soil properties was found compared to the plant properties and composition of plant root exudates. The small effect exerted by plant species was not direct, but instead mediated through the modulation of exudation, and it is only discernible when the effects of soil type are removed. The small influence of plant species on rhizosphere bacterial communities and the similarity in root exudate composition between plants likely translate the effects of the tight spatial arrangement of plant roots in natural settings, in which rhizosphere areas of multiple plants overlap, leading to the development of highly similar bacterial communities. This represents a novel perspective into the rhizosphere since most previous investigations report the existence of various degrees of plant influence.

Another novel insight into community establishment arises from the observation that soil mineral properties have a big influence in bacterial communities. The observed consistent temporal patterns of successional suggests that de novo inserted minerals in soils have a strong deterministic effect on the establishment of bacterial communities. This influence seems larger than the effect of the distinct food sources with which minerals were loaded, which is surprising since nutrient composition and quality are major bacterial drivers. Moreover, the successional changes could be attributed to specific bacteria, but did not indicate of taxonomical coherence. It was evident that closely related bacteria can have distinct responses and therefore more investigations at higher taxonomic resolution are needed for the prediction of early colonization of mineral surfaces.

The use of culture dependent techniques targeting oligotrophic soil bacteria enabled the retrieval of a large number of novel isolates. The information gathered not only assists in selecting and improving cultivation strategies, but the isolates obtained provide important information on their ecological role in the environment. Some of the novel bacteria retrieved were identified as having a prominent role in both rhizosphere and on the colonization of minerals and may prove useful in understanding the complex interactions occurring in these environments. Additionally, even when

found rare in the environments in the scope of the present work, the novel bacteria isolated are abundant and can inform over other environments.

The knowledge gathered in the present work sheds light on the drivers of bacterial communities in distinct natural soil environments. Nevertheless, further work is required in order to validate the new perspectives suggested for natural environments, such as the analysis of the plant effects on rhizosphere bacteria in other grassland types (savannah, steppes, etc.), and restored grassland ecosystems. Also of interest would be to account for the effect of plants in affecting the colonization of mineral surfaces, since the bacterial community changes in soils under formation is thought to parallel aboveground vegetation. This would allow to further disentangle the variability in microbial communities within soils and their response to environmental contexts.

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Curriculum vitae

Personal Data

Name - Selma Gomes Vieira
Nationality - Portuguese
Place of Birth – Leiria, Portugal
Date of Birth - 3rd July 1987

Academic Formation

May 2014 – June 2018	**Doctoral studies** Microbial ecology and diversity Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures
September 2010 – May 2012	**Master degree** Comparative, Tecnological and Molecular Genetics University of Trás-os-Montes and Alto Douro
September 2005 – July 2010	**Bachelor degree** Genetics and Biotechnology University of Trás-os-Montes and Alto Douro
September 2002 – July 2005	**Secondary School** Area of Science and Technology Colégio de São Miguel, Fátima, Portugal.

Previous scientific activities

May 2014 to June 2018	**PhD dissertation** *Bacterial adaptation strategies and interactions in different soil habitats* Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures (Professor Dr. Jörg Overmann)
September 2013 to February 2014	**Research fellow** Project: *Bax activation by PKCε modulation: an approach to eliminate cancer cells* Centre of Molecular and Environmental Biology of the University of Minho (CBMA-UM) (Professor Dr. Susana Chaves)
May 2012 to August 2013	**Volunteer** Project: *Phosphoregulation of Bax-dependent cell death* Centre of Molecular and Environmental Biology of the University of Minho (CBMA-UM) (Professor Dr. Manuela Côrte-Real)

September 2010 to July 2011	**Masters dissertation** *Genetic characterization of virulence factors and protein profile analysis of Enterococcus spp. strains, isolated from seagulls Larus cachinnans* Center of Animal and Veterinary Sciences of the University of Trás-os-Montes and Alto Douro (CECAV-UTAD) (Professor Dr. Patrícia Poeta)
April 2009 to March 2010	**Scholarship for Research Integration** Project: *Genetic and protein characterization of antibiotic resistance in strains of Escherichia coli and Enterococcus spp. from consumption animals (bovines, small ruminants and swines)* Center of Animal and Veterinary Sciences of the University of Trás-os-Montes and Alto Douro (CECAV-UTAD) (Professor Dr. Patrícia Poeta)
March 2008 to July 2008	**Bachelor degree dissertation** *Proteomic analysis of Salmonella and Staphylococus aureus through mono and bidimensional electrophoresis* Centre of Genetics and Biotechnology of the University of Trás-os-Montes and Alto Douro (CGB-UTAD) (Professor Dr. Gilberto Igrejas)
October 2006 to June 2007	**Volunteer in the area of plant molecular genetics** Centre of Genetics and Biotechnology of the University of Trás-os-Montes and Alto Douro (CGB-UTAD) (Professor Dr. Maria Manuela Matos)

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